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EDITING
Editorial Board of *World Journal of Gastroenterology,* 77 Shuangta Xijie, Taiyuan 030001,
Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com

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LIVER CANCER

Chinese medicinal compound delisheng has satisfactory anti-tumor activity, and is associated with up-regulation of endostatin in human hepatocellular carcinoma cell line HepG2 in three-dimensional culture

Jie Cui, Ke-Jun Nan, Tao Tian, Ya-Huan Guo, Na Zhao, Lin Wang

Jie Cui, Ke-Jun Nan, Tao Tian, Ya-Huan Guo, Na Zhao, Lin Wang, Department of Medical Oncology, The First Affiliated Hospital of The School of Medicine of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Correspondence to: Ke-Jun Nan, Department of Medical Oncology, The First Affiliated Hospital of The School of Medicine of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. nankj@163.com
Telephone: +86-29-85324086 Fax: +86-29-85324086
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Abstract

AIM: To investigate the multicellular resistance of human hepatocellular carcinoma HepG2 cells in three-dimensional culture to delisheng, 5-fluorouracil and adriamycin, and the possible molecular mechanisms of delisheng.

METHODS: Human hepatocellular carcinoma HepG2 cells were cultured with a liquid overlay technique. After the formation of multicellular spheroids, morphology was analyzed by phase contrast microscopy, scanning electron microscopy and transmission electron microscopy. Sensitivity of HepG2 cells to delisheng, 5-fluorouracil and adriamycin was investigated by MTT assay in multicelluar spheroids and monolayers. Vascular endothelial growth factor (VEGF) and endostatin expression were analyzed in multicellular spheroids treated with delisheng, 5-fluorouracil, adriamycin and negative control PBS, with immunohistochemical staining.

RESULTS: Multicellular spheroids exhibited structural characteristics somewhat different to those in monolayers. The cells in three-dimensional cell culture turned out to be less sensitive to delisheng, 5-fluorouracil and adriamycin than the cells cultured in monolayer. This showed that delisheng had a satisfactory cells inhibition ratio compared to 5-fluorouracil and adriamycin. Immunohistochemical staining showed that VEGF and endostatin expression was positive during growth as multicellular spheroids, and endostatin expression in spheroids with treatment of delisheng was higher than that with 5-fluorouracil, adriamycin and PBS (139.35 ± 7.83, 159.23 ± 10.34, 162.83 ± 3.47 and 148.48 ± 11.06, $P < 0.05$).

CONCLUSION: Chinese medicine compound delisheng has satisfactory anti-tumor activity in HepG2 cells in three-dimensional culture, and the effects are associated with up-regulation of endostatin.

Key words: Delisheng; Ginseng; Three-dimensional culture; Multicellular resistance; Endostatin

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a very high morbidity and mortality, and a poor prognosis. Its incidence is increasing both in Asian countries and in the USA. A majority of HCC patients presents with advanced or unresectable disease. Even for those with resected disease, the recurrence rate can be as high as 50% at 2 years. Despite extensive efforts by many investigators, systemic chemotherapy for HCC has been quite ineffective, as demonstrated by low response rates and no survival benefits[1-4]. With the continuous development of the traditional Chinese medicine industry in recent years, it has been proven that traditional Chinese medicines (TCMs) have a marked effect on treating HCC, with unique advantages, and have gained wide acceptance as a safe, palliative and effective treatment in China[5-9]. Delisheng is a Chinese medicinal compound and is usually used in combination with chemotherapy for HCC. Furthermore, it has been reported that it can improve the clinical symptoms and quality of life, without severe adverse reactions, in patients with late-stage HCC. It is composed of ginseng, milk vetch root, secretion bufonis and Cantharidium. As delisheng is attractive as a natural product for medicinal use, increasing attention is being

paid to its scientific evaluation and its possible molecular mechanisms.

Three-dimensional cell culture has been widely used for studying the various molecular processes and the development of therapy in recent years, for subtle changes in phenotypic expression and biological activity not demonstrated in conventional monolayer culture. In contrast, multicellular spheroids of tumor cells provide an excellent three-dimensional *in vitro* model to facilitate detailed investigations, including the response to various antineoplastic agents and their possible molecular mechanisms, since spheroids mimic the solid tumors more closely than monolayers do[10]. Tumor resistance to anticancer drugs is a real phenomenon, partly because of the so-called multicellular resistance (MCR), and it may be the most important obstacle to cancer treatment[11]. The resistance encountered in cells cultured as spheroids seems to be analogous to the natural resistance observed in patients, so the usage of three-dimensional cell culture may provide a model for studies on the development of anti-cancer drugs.

In this study, cells were cultured with a liquid overlay technique[12,13]. After the formation of multicellular spheroids, morphology was analyzed by phase contrast microscopy, scanning electron microscopy and transmission electron microscopy. Sensitivity of human hepatocellular carcinoma HepG2 cells to delisheng, 5-fluorouracil and adriamycin was investigated by MTT assay in multicelluar spheroids and monolayers. Vascular endothelial growth factor (VEGF) and endostatin expression was analyzed in multicellular spheroids treated with delisheng, 5-fluorouracil, adriamycin and negative control PBS, with immunohistochemical staining.

MATERIALS AND METHODS

Human hepatocellular carcinoma cell line

The human hepatocellular carcinoma cell line used in the present study was HepG2 preserved in The Center of Molecular Biology of Xi'an Jiaotong University.

Monolayer and three-dimensional cell cultures

Each HepG2 cell line was maintained in DMEM (Gibco, USA) medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin in 5% CO_2/95% air at 37℃. Cell cultures were maintained in the exponentially growing state by passaging twice weekly. Exponentially growing cells were harvested in a monolayer cell culture, while for three-dimensional cell culture obtained by liquid overlay technique, a single cell suspension in complete medium was seeded in each culture flask coated with 2% agarose. The conditions for three-dimensional cell culture were exactly the same as for monolayer culture, except for the presence of an agarose layer. After 3 or 4 d incubation, multicellular spheroids were obtained from each culture flask.

Scanning electron microscopy

After 3 or 4 d culture, monolayer cells and multicellular spheroids were observed by phase contrast microscopy. After which, samples were washed with PBS, pH 7.4, and fixed with 2.5% glutaraldehyde for 2 h at 4℃. After three washes in PBS, they were post-fixed with 1.0% osmium tetroxide in PBS for 2 h at 4℃, then washed once in PBS, followed by dehydration with an increasing ethanol series (30, 50, 70, 90 and 100%). The samples were then treated with isoamyl acetate for 10 min, dried to the critical point, and coated with gold. Finally, the samples were observed with a scanning electron microscope (JEOL, JSM-840, Japan).

Transmission electron microscopy

Additional samples were fixed and dehydrated as described for scanning electron microscopy, and embedded in Epon812 epoxy resin. Thin sections were prepared and examined with a transmission electron microscope (HITACHI, H-600, Japan).

MTT assay

The Chinese medicinal compound delisheng was dispensed into a physic liquor to give a clinical dosage of 80 mL (recommended dose is 50-100 mL/d). Delisheng was attenuated with Hanks balanced salts solution, and the final concentration was 12.5, 25, 50, 100 and 200 μL/mL. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma, St. Louis, MO, USA) was dissolved in PBS at 5 mg/mL and sterilized by filtration. After treatment with 12.5, 25, 50, 100 or 200 μL/mL delisheng; 6.25×10^{-3}, 12.5×10^{-3}, 25×10^{-3}, 50×10^{-3} and 100×10^{-3} g/L 5-fluorouracil; 0.15625×10^{-3}, 0.3125×10^{-3}, 0.625×10^{-3}, 1.25×10^{-3} and 2.5×10^{-3} g/L adriamycin in monolayer and three-dimensional cell culture for 48 h, the cells were freshly disaggregated by enzymatic dissociation, and the cell number was determined with a hemocytometer. A cell suspension (150 μL) of each sample was added to a 96-well plate. The cell number per well was approximately 2×10^5, Each well was added to stock MTT solution (20 μL, 5 mg/mL). After incubation in the presence of 5% CO_2 and 95% air at 37℃ for 4 h, the supernatant was discarded. DMSO (150 μL) (Sigma) was added to each well and mixed thoroughly to dissolve the dark blue crystals. After 30 min at room temperature to ensure that all crystals were dissolved, the plates were read with a Micro Elisa plate reader at a wavelength of 492 nm. All samples were read five times. The cell inhibition rate was calculated by the following formula: cell inhibition rate (%) = (1-OD of treated cells)/(OD of control cells) × 100%.

Immunohistochemical staining

Antibody staining was performed with multicellular spheroids. The monoclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:25. The monoclonal anti-endostatin (Santa Cruz Biotechnology) was used at a dilution of 1:25. Spheroids treated with delisheng (25 μL/mL), 5-fluorouracil (40×10^{-3} g/L), adriamycin (1×10^{-3} g/L) and negative control PBS for 48 h were washed in PBS, and fixed in 4% paraformaldehyde in PBS at 4℃ for 1 h, placed in a small tissue processing cassette full of 3%

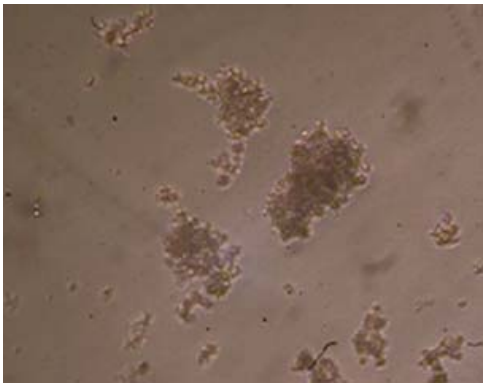

Figure 1 Multicellular spheroids of hepG2 cells observed with the phase contrast microscope (× 100).

Figure 2 Monolayer of hepG2 cells observed with the phase contrast microscope (× 100).

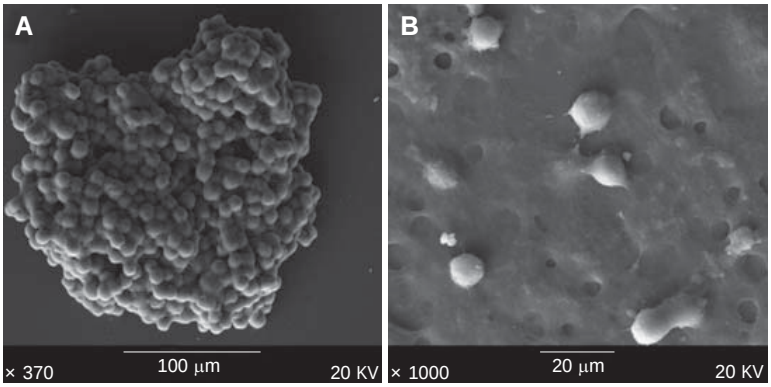

Figure 3 Scanning electron microscopy of hepG2 cells. **A**: The multicellular sphere was irregular with a diameter up to 1.0-2.0 mm, tight cell junctions were observed; **B**: Monolayer cells spread dispersively and cell junctions were hardly observed.

agarose for 1 h, then dehydrated and embedded in paraffin. Four-micrometer sections from the representative blocks were immunohistochemically stained with mouse anti-human monoclonal antibodies against VEGF, and rabbit anti-human monoclonal antibodies against endostatin. Four-micrometer thick sections were deparaffinized, rehydrated and washed in PBS for 15 min, before endogenous peroxidase activity was blocked. Primary antibody was substituted by normal mouse and rabbit serum as a negative control .Then endogenous peroxidase activity was blocked by 30 min incubation in 3% hydrogen peroxide solution. The specimens were washed with PBS, pH 7.5. Non-specific binding was blocked by incubating the slides with normal goat serum in PBS for 15 min at 37℃, then incubated overnight at 4℃ with the primary antibodies. After washing three times with PBS, the sections were incubated with secondary antibody, biotinylated antibodies for 40 min at 37℃. After washing three times with PBS, the sections were immunostained with avidin-biotin complex for 40 min at 37℃. Visualization of the immunoreactions was conducted with 3, 3'-diaminobenzidine (DAB, Sigma, UK) for 5 min. Finally, sections were counterstained with hematoxylin. The degree of the expression of immunohistochemical products was classified into negative (< 10% of cells had a positive reaction) and positive (> 10% of cells had a positive reaction). Simultaneously, VEGF- and endostatin-positive staining particles were quantitatively analyzed by a LeicaQ550cw imaging analysis system (Germany) to determine the mean grey values, the lower mean grey values, and the stronger substrate coloration. The mean grey values are an inverse ratio with the protein expression quantity.

Statistical analysis

Data were reported as means ± SE. The *t* test was used for statistical analysis, $P < 0.05$ was considered statistically significant.

RESULTS

Cell morphology (phase contrast microscopy, scanning and transmission electron microscopy)

Multicellular spheroids and monolayer cells were observed with phase contrast microscopy. The cells were oval spheroids in three-dimensional cell culture (Figures 1 and 2).

Scanning electron microscopy showed that the multicellular spheroids were irregular, with a diameter of up to 200 μm at 3-4 d. The cells were oval spheroids or polyhedrons, with tight cell junctions, compared to monolayer cells that were spread in a dispersed manner and had few cell junctions (Figure 3).

Transmission electron microscopy showed that desmosome and intermediate junctions were observed in three-dimensional cell culture, but such structures were rarely observed in monolayer cell culture. Multicellular spheroids of approximately 200 μm diameter showed no obvious signs of extensive central apoptosis or necrosis,

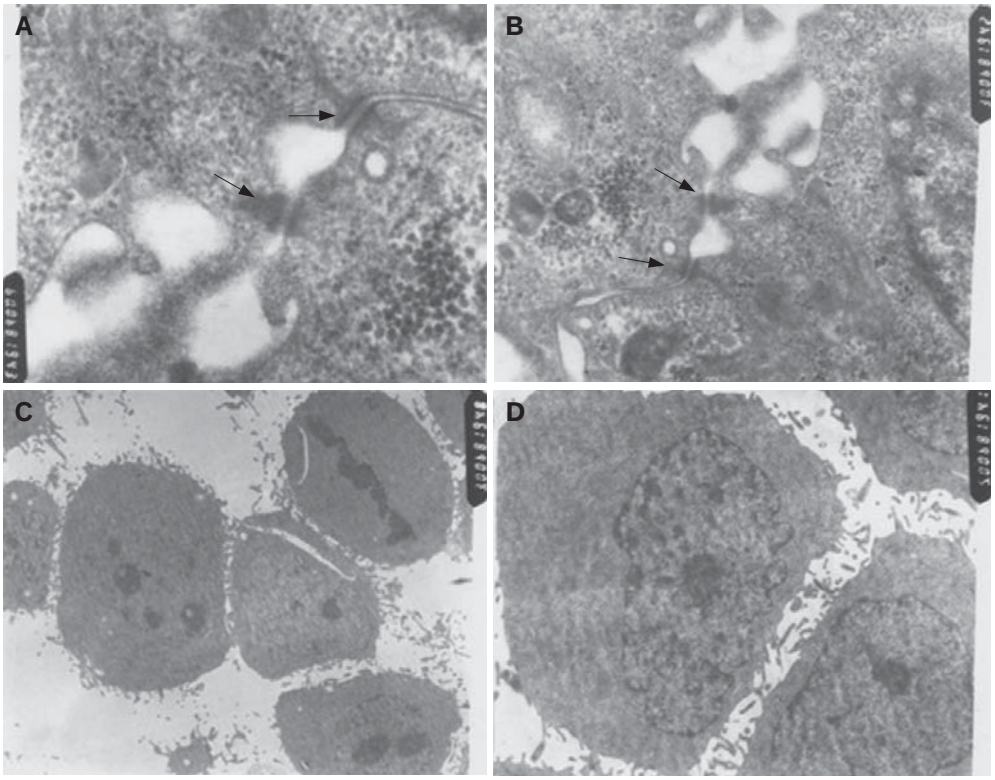

Figure 4 Transmission electron microscopy of hepG2 cells: **A** (× 60 000); **B** (× 30 000): desmosome junctions and intermediate junctions were observed in three-dimensional cell culture; **C** (× 3000); **D** (× 5000): cell junctions were hardly observed in monolayer cells with more microvilli on the surfaces.

Table 1 Test sensitivity of delisheng in 3d and 2d cell culture

Concentration (μL/mL)	Inhibition ratio (%)		*P* value
	3-d	Monolayer	
12.5	11.73 ± 11.58	33.81 ± 10.54	0.014
25	20.94 ± 8.29	37.79 ± 9.55	0.018
50	25.45 ± 5.62	46.01 ± 6.32	0.001
100	36.69 ± 5.37	75.65 ± 10.47	< 0.001
200	43.93 ± 7.81	84.62 ± 3.24	< 0.001

Table 2 Test sensitivity of 5-fluorouracil in 3d and 2d cell culture

Concentration (g/L)	Inhibition ratio (%)		*P* value
	3-d	Monolayer	
6.25×10^{-3}	18.25 ± 7.85	33.89 ± 9.41	0.021
12.50×10^{-3}	17.51 ± 7.28	39.08 ± 7.74	0.002
25.00×10^{-3}	18.18 ± 11.34	43.18 ± 5.92	0.002
50.00×10^{-3}	17.87 ± 10.93	67.72 ± 2.18	< 0.001
100.00×10^{-3}	36.42 ± 10.54	81.17 ± 2.81	< 0.001

although there was some swelling and there were fewer microvilli on the surface of multicellular spheroids compared to monolayer cells (Figure 4).

Response to delisheng, 5-fluorouracil and adriamycin exposure

The sensitivity of monolayer and three-dimensional cell cultures to delisheng, 5-fluorouracil and adriamycin was investigated by MTT assay. The cell inhibition ratio in three-dimensional cell culture treated with delisheng, 5-fluorouracil and adriamycin was lower than that in monolayer culture ($P < 0.01$) (Tables 1-3, Figures 5-7), which indicated the HepG2 cells in three-dimensional culture became resistant to delisheng, 5-fluorouracil and adriamycin. Cell inhibition ratio increased with concentration of delisheng, 5-fluorouracil and adriamycin. The results showed that delisheng had a satisfactory cell inhibition ratio compared to 5-fluorouracil and adriamycin.

Table 3 Test sensitivity of adriamycin in 3d and 2d cell culture

Concentration (g/L)	Inhibition ratio (%)		*P* value
	3-d	Monolayer	
0.15625×10^{-3}	11.40 ± 2.71	10.39 ± 5.07	0.705
0.31250×10^{-3}	12.17 ± 6.49	21.31 ± 2.85	0.02
0.62500×10^{-3}	18.61 ± 9.41	25.69 ± 8.19	0.24
1.25000×10^{-3}	29.45 ± 5.26	41.66 ± 6.52	0.012
2.50000×10^{-3}	38.09 ± 20.71	84.56 ± 7.97	0.002

Data were reported as means ± SE ($n = 5$).

Immunohistochemistry staining

VEGF and endostatin protein expression were confirmed in multicellular spheroids in three-dimensional culture. Immunohistochemical analysis demonstrated that VEGF and endostatin were expressed in the cytoplasm. Endostatin expression in spheroids treated with delisheng was higher than that with 5-fluorouracil, adriamycin and negative control PBS ($P < 0.05$). However, VEGF expression in spheroids treated with delisheng was similar with 5-fluorouracil, adriamycin and PBS ($P > 0.05$) (Tables 4 and 5; Figure 8).

DISCUSSION

HCC is one of the most common types of human

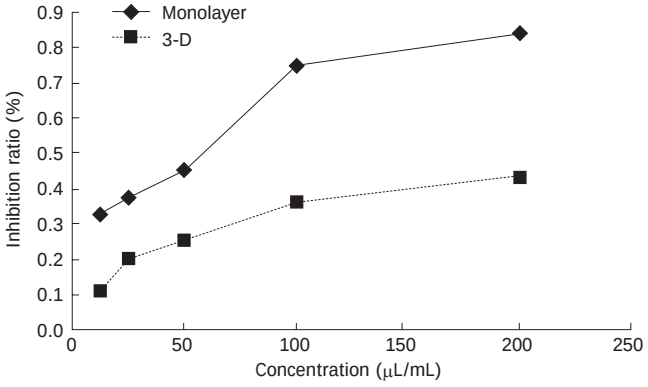

Figure 5 Sensitivity of HepG2 cells to delisheng determined by the MTT assay. After the treatment with 12.5, 25, 50, 100 and 200 μL/mL delisheng for 48 h, the cells in three-dimensional cell culture and monolayer culture were cultured with MTT solution and cell inhibition ratio was determined. The cells in monolayer culture were more sensitive to delisheng ($P < 0.01$).

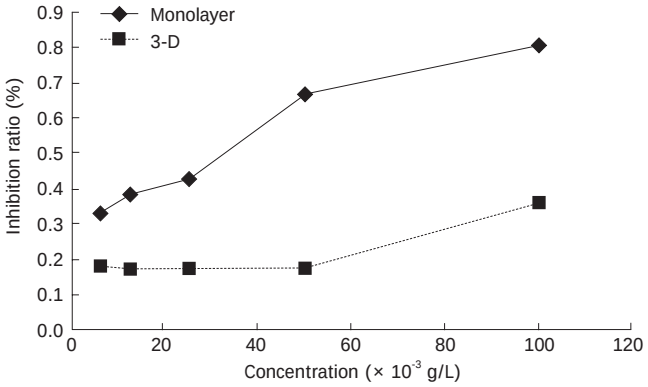

Figure 6 Sensitivity of HepG2 cells to 5-fluorouracil determined by the MTT assay. After the treatment with 6.25, 12.5, 25, 50 and 100 × 10^{-3} g/L 5-fluorouracil for 48 h, the cells in three-dimensional cell culture and monolayer culture were cultured with MTT solution and cell inhibition ratio was determined. The cells in monolayer culture were more sensitive to 5-fluorouracil ($P < 0.01$).

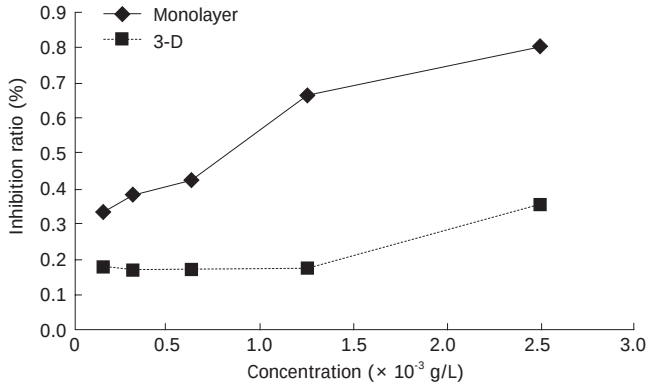

Figure 7 Sensitivity of HepG2 cells to adriamycin determined by the MTT assay. After the treatment with 0.15625, 0.31250, 0.62500, 1.25000, 2.50000 × 10^{-3} g/L adriamycin for 48 h, the cells in-three dimensional cell culture and monolayer culture were cultured with MTT solution and cell inhibition ratio was determined. The cells in monolayer culture were more sensitive to adriamycin ($P < 0.01$).

Table 4 Endostatin expression of multicellular spheroids with treatment of delisheng, 5-fluorouracil, adriamycin and PBS

Drug	Mean grey value
Delisheng	139.35 ± 7.83
5-fluorouracil[a]	159.23 ± 10.34
Adriamycin[c]	162.83 ± 3.47
PBS[e]	148.48 ± 11.06

[a]$P < 0.05$ 5-fluorouracil *vs* delisheng, [c]$P < 0.05$ adriamycin *vs* delisheng, [e]$P < 0.05$ PBS *vs* delisheng.

Table 5 VEGF expression of multicellular spheroids with treatment of delisheng, 5-fluorouracil, adriamycin and PBS

Drug	Mean grey value
Delisheng	188.00 ± 6.33
5-fluorouracil[a]	189.93 ± 16.58
Adriamycin[c]	193.44 ± 5.11
PBS[e]	184.82 ± 13.87

[a]$P > 0.05$ 5-fluorouracil *vs* delisheng, [c]$P > 0.05$ adriamycin *vs* delisheng, [e]$P > 0.05$ PBS *vs* delisheng.

malignancy worldwide. The prognosis in patients with untreated HCC is very poor, with a median survival of 6 mo in patients who receive no specific treatment[14]. Curative therapy such as surgery, liver transplantation[15], or percutaneous treatments benefit only 25% of patients. Systemic chemotherapy has been widely used in an attempt to prolong this short survival time or to provide symptomatic relief[16], but it appears to be less efficient, possibly because it is used when metastases are already present, and the tumor is large and spreading; moreover, anticancer drug resistance is frequent. Tumor resistance to anticancer drugs is a real phenomenon, but the most prevalent mechanism may not correspond to multidrug resistance[17]. Instead, the so-called MCR, first described in 1972 by Durand and Sutherland may be the most important obstacle to cancer treatment. The resistance encountered in cells cultured as spheroids seems to be analogous to the natural resistance observed in patient tumors. Tumor cells often form compact multicellular spheroids when maintained in a three-dimensional culture system. Various changes in molecular expression and even in biological activity have been reported to exist between the three-dimensional and conventional monolayer cultures[18,19].

There are abundant resources in TCMs that have been used clinically for > 5000 years in China and Asia, and increasing attention is being paid to their scientific evaluation. With continuing development of TCM, it has a marked effect on the treatment of several, including tumors, with unique advantages. Delisheng is a common Chinese medicinal compound, whose composition includes ginseng, milk vetch root, secretion bufonis and Cantharidium. Satisfactory effects of delisheng have been reported in patients with late-stage HCC that may improve clinical symptoms and quality of life, without severe adverse reactions. However, the mechanisms responsible for this treatment are unknown. Many kinds of solid tumors *in vivo* and tumor cells in three-dimensional cell culture *in vitro* exhibit intrinsic or acquired resistance to

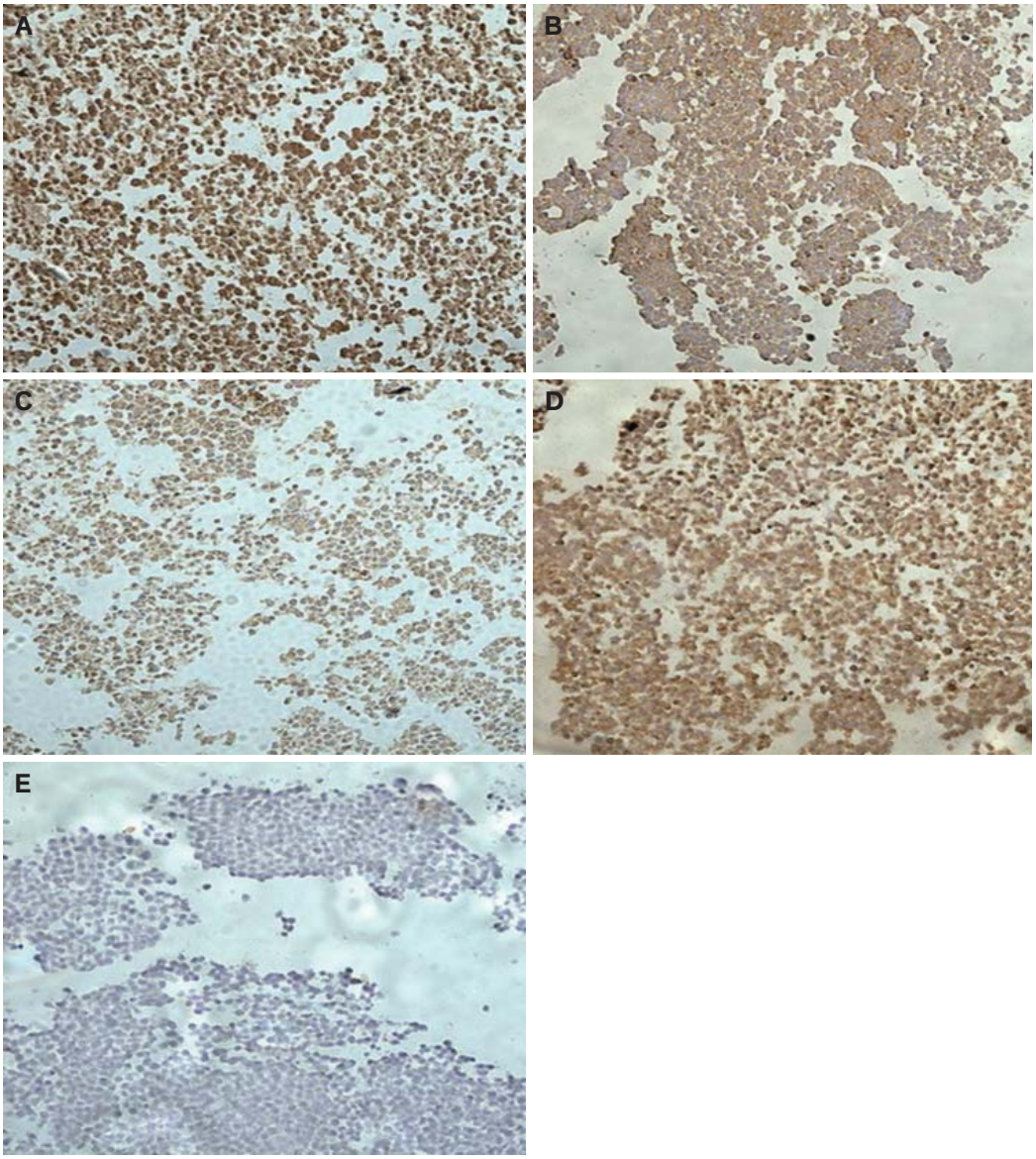

Figure 8 Immunohistochemical staining patterns of formalin-fixed and paraffin-embedded multicellular spheroids of HepG2 cells (× 200) [**A**: delisheng; **B**: adriamycin; **C**: 5-fluorouracil; **D**: negative control PBS; **E**: negative control (Primary antibody was substituted by normal rabbit serum)]. Endostatin expression was confirmed in multicellular spheroids, and the expression of endostatin with treatment of delisheng was higher than that of 5-fluorouracil, adriamycin and PBS ($P < 0.05$).

cytotoxic drugs, which is one of the major obstacles to clinical treatment.

In this study, human HepG2 cells were cultured with a liquid overlay technique to form multicellular spheroids. The results indicated that the cells were oval spheroid or polyhedral, with fewer microvilli on the surface and more desmosome and intermediate junctions, compared to monolayer cells. The cells in three-dimensional culture turned out to be less sensitive to delisheng, 5-fluorouracil and adriamycin than those cultured in monolayer. Some studies have indicated that more cells in multicellular spheroids shift into a quiescent state. However, the majority of conventional cytotoxic anticancer drugs preferentially kill cycling cells. The increase in quiescent cells might result in decreased sensitivity. VEGF and endostatin expression were confirmed in three-dimensional culture. The data indicated that endostatin expression in spheroids treated with delisheng was higher than that with 5-fluorouracil and adriamycin and negative control PBS, and a previous study has shown that delisheng has a satisfactory cell inhibition ratio compared to 5-fluorouracil and adriamycin. This suggests that the satisfactory effects of delisheng on HCC were associated with the up-regulation of endostatin. As we know, one of the components of delisheng, ginseng, has some antiangiogenic activity. Recent studies have reported that ginseng extract exerts anti-tumor activity through its effect on the vascular system; furthermore, some investigators have suggested that ginsenoside Rg3, a saponin extracted from ginseng, alone or combined with cyclophosphamide (CTX), inhibits growth and angiogenesis of ovarian cancer by decreasing the microvessel density (MVD value) and VEGF expression[20-23]. Endostatin as an angiogenesis inhibitor has been shown to inhibit VEGF-induced endothelial cell migration *in vitro*, and to have anti-tumor activity *in vivo*[24,25]. Some investigators have studied tumor growth in transgenic mice overproducing endostatin specifically in the endothelial cells (a 1.6-fold increase in the circulating levels), and found that tumor growth was 3-fold slower than in wild-type mice[26-30]. Therefore, we think that the endostatin up-regulation induced by delisheng in our experiment was possibly due to the antiagiogenic activity of its ginseng component, and this may explain why delisheng has satisfactory anti-tumor

activity too. Although major emphasis has been placed on the down-regulation of VEGF, the potential role of endostatin increase induced by ginseng as an endogenous inhibitor of angiogenesis in tumor growth inhibition can not be ignored.

Further understanding of the mechanisms involved in the activity of delisheng will help in the development of new approaches to therapy of HCC. The satisfactory activity of delisheng on HCC was associated with ginseng up-regulation of endostatin expression.

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COMMENTS

Background

HCC is a highly malignant tumor with a very high morbidity and mortality. Despite extensive efforts by many investigators, systemic chemotherapy for HCC has been quite ineffective. Delisheng is a Chinese medicinal compound and is often used in conjunction with chemotherapy for HCC, with satisfactory results. Our work tried to establish the mechanisms for these effects of delisheng on HCC. Three-dimensional cell culture has been widely used for studying the various molecular processes, since spheroids mimic solid tumors more closely than monolayers do, so the use of three-dimensional culture provides a model for the development of anti-cancer drugs. In this study, cells were cultured with a liquid overlay technique. After the formation of multicellular spheroids, we used the model to perform our experiments.

Research frontiers

With the continuous development of TCM in recent years, it is been demonstrated that it can have a marked effect on treating HCC, with unique advantages, and it has gained wide acceptance as a safe, palliative and effective treatment in China. Delisheng is a Chinese medicinal compound and is often used in conjunction with chemotherapy for HCC. Furthermore, it has been reported in patients with late-stage HCC that delisheng may improve the clinical symptoms and quality of life, without severe adverse reactions. As delisheng is attractive as a natural product for medicinal use, increasing attention is being paid to its scientific evaluation and its possible molecular mechanisms. Three-dimensional cell culture has been widely used for studying the various molecular processes and development of therapy in recent years, as it detects subtle changes in phenotypic expression and biological activity not seen in conventional monolayer culture. In contrast, multicellular spheroids of tumor cells provide an excellent three-dimensional *in vitro* model to facilitate detailed investigations, including the response to various antineoplastic agents and their possible molecular mechanisms, since spheroids mimic solid tumors more closely than monolayers do. The resistance encountered in cells cultured as spheroids seems to be analogous to the natural resistance observed in patient tumors, so the usage of three-dimensional cell culture may provide a model for developing anti-cancer drugs.

Innovations and breakthroughs

We used three-dimensional cell culture to study a Chinese medicine and its anti-cancer effects. We showed that delisheng had satisfactory anti-cancer effects on HCC, and these were associated with the up-regulation of endostatin. This was possible because of the presence of ginseng in delisheng.

Applications

We confirmed that three-dimensional cell culture was suitable for the study of a traditional Chinese medicine, and this may help other researchers to find a better model for drug development. We also found that delisheng had satisfactory anti-cancer effects on HCC, and these were associated with the up-regulation of endostatin. This was made possible by one of delisheng's components, ginseng, and this may provide a new method of therapy for HCC.

Terminology

Three-dimensional cell culture: this has been widely used for studying the various molecular processes and development of therapy in recent years, because it can detect subtle changes in phenotypic expression and biological activity not seen in conventional monolayer culture. This is because spheroids mimic solid tumors more closely than monolayers do. Delisheng: a Chinese medicinal compound that is often used in conjunction with chemotherapy for HCC. Furthermore, it has been reported in patients with late-stage HCC that it can improve clinical symptoms and quality of life, without severe adverse reactions. Its composition includes ginseng, milk vetch root, secretion bufonis and Cantharidium.

Peer review

The article provides a new model to study TCM, and explains the test outcome rationally; furthermore, it introduces the Chinese medicinal compound delisheng and indicates its further applications.

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EDITORIAL

Genetic epidemiology of primary sclerosing cholangitis

Tom H Karlsen, Erik Schrumpf, Kirsten Muri Boberg

Tom H Karlsen, Erik Schrumpf, Kirsten Muri Boberg, Medical Department, Rikshospitalet-Radiumhospitalet Medical Center, Oslo, Norway
Correspondence to: Dr. Tom H Karlsen, Medical Department, Rikshospitalet-Radiumhospitalet Medical Center, N-0027 Oslo, Norway. t.h.karlsen@klinmed.uio.no
Telephone: + 47-23074226 Fax: +47-23073510
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Abstract

The aetiology of primary sclerosing cholangitis (PSC) is not known. A more than 80-fold increased risk of PSC among first-degree relatives emphasizes the importance of genetic factors. Genetic associations within the human leukocyte antigen (HLA) complex on chromosome 6p21 were detected in PSC 25 years ago. Subsequent studies have substantiated beyond doubt that one or more genetic variants located within this genetic region are important. The true identities of these variants, however, remain to be identified. Several candidate genes at other chromosomal loci have also been investigated. However, according to strict criteria for what may be denominated a susceptibility gene in complex diseases, no such gene exists for PSC today. This review summarises present knowledge on the genetic susceptibility to PSC, as well as genetic associations with disease progression and clinical subsets of particular interest (inflammatory bowel disease and cholangiocarcinoma).

Key words: Primary sclerosing cholangitis; Genetic associations; Human leukocyte antigens; Cholangiocarcinoma; Inflammatory bowel disease

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic inflammatory condition of unknown aetiology, characterised by progressive strictures of the intra- and extrahepatic bile ducts and eventually liver cirrhosis and liver failure[1,2]. No effective medical treatment is currently available[3,4], and PSC is the major indication for liver transplantation in the Scandinavian countries as well as the fifth leading indication for liver transplantation in the United States[5,6]. Population-based studies of disease frequency are available from Norway, Great Britain and The United States[7-9], and indicate comparable incidence (0.9-1.3 per 100 000/year) and prevalence (8.5-14.2 per 100 000) rates for these populations. The prevalence of PSC is probably lower in Southern European and Asian populations[10]. In contrast to the female predominance of many autoimmune diseases, approximately 2/3 of the PSC patients are male[11]. Affected individuals are young (less than 40 years at time of diagnosis), and median survival from time of diagnosis by cholangiography to death or liver transplantation is approximately 12 years[11].

Up to 80% of the PSC patients of Northern European origin have concurrent inflammatory bowel disease (IBD)[10]. The frequency in Southern Europe and Asia is lower (around 50% and 35%, respectively)[12-14]. According to standard criteria[15], the IBD phenotype in PSC has mainly been classified as ulcerative colitis (UC), although an association with colonic Crohn's disease also exists[16,17]. The increased frequency of a variety of other autoimmune diseases (e.g. type 1 diabetes) among patients with PSC does not seem related to the increase in IBD[18]. There is also an increased risk of cancer among the patients with PSC, not only cholangiocarcinoma of the biliary tract (approximately 13%-14% in Scandinavia)[19,20], but also other gastrointestinal malignancies (i.e. pancreatic and colorectal cancer)[19]. The diagnosis of cholangiocarcinoma is difficult because the cholangiographic changes may look similar to those found in PSC without cholangiocarcinoma[21]. As a result, the cancer is often recognised at an advanced stage when treatment by liver transplantation does not improve survival[22].

Smoking is the only environmental factor known to influence PSC susceptibility and is associated with a reduced risk of the disease[23]. Several genetic risk factors, however, have been repeatedly described throughout the 25 years since they were first detected[24,25]. The present editorial aims to summarise present knowledge on statistical associations between genetic variants and risk of PSC or particular characteristics of PSC. In genetic epidemiology, disease characteristics under study are called phenotypes. Etymologically, the pheno-prefix refers to "visible" or "evident". Phenotypes, also referred to as traits, may be dichotomous (e.g. PSC/healthy) or quantitative (e.g. the level of alkaline phosphatase in a blood sample from a PSC patient). The clinical definition of a disease is primarily made to decide whether a

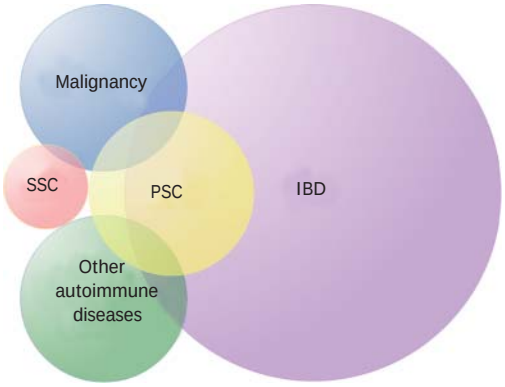

Figure 1 Primary sclerosing cholangitis (PSC) is a patchwork of different phenotypes in addition to the bile duct involvement. Most important are inflammatory bowel disease (IBD), malignancy and other autoimmune diseases. PSC is distinct from secondary sclerosing cholangitis (SSC).

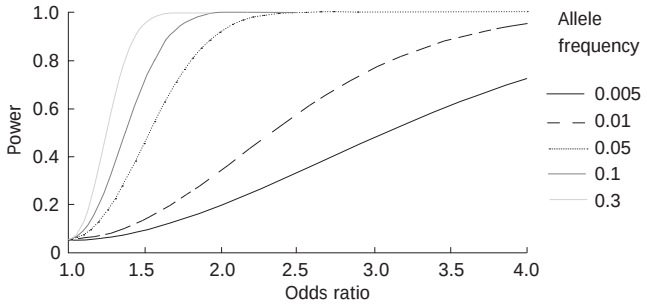

Figure 2 Statistical power (α = 0.05) for different odds ratios and allele frequencies in a study of 365 patients and 365 controls, i.e. the number of alleles in each group is 2 n = 730.

particular treatment or follow-up may be indicated for a patient or not. This practical aspect means that PSC as a clinical "diagnosis" does not necessarily equal the ideal "phenotype" for genetic association studies. The disease phenotype in such studies should be as homogeneous as possible, simply because the presence of irrelevant phenotypes in a study population will reduce the strength of effects to be identified. The clinical phenotype of PSC is compound (Figure 1).

In other diseases, susceptibility genes have been identified through genome-wide linkage scans followed by fine-mapping[26-28]. In PSC, the lack of families with affected sibling pairs has not allowed such studies to position susceptibility loci[28]. The search for PSC susceptibility genes has thus focused on plausible candidates with regard to function[25]. As a general basis for interpreting candidate gene association studies, an introduction to important concepts of such studies will be given, followed by a presentation and discussion of studies performed in PSC. We searched PubMed for relevant articles published up until the end of April 2007. We have also reviewed the reference lists of identified articles, as well as the reference lists of major immunogenetic- and hepatology conferences held over the last 2 years.

GENETIC CONSIDERATIONS

In genetic terms, PSC is considered a complex trait, meaning that polymorphisms in several genes along with environmental factors are required for disease development[27]. Heritability for a disease is measured by (a) concordance rates in monozygotic versus dizygotic twins and (b) relative risk in siblings of a patient (λ_s = prevalence among siblings divided by the general population prevalence). For monogenic disorders, λ_s ranges from several hundreds to several thousands, whereas values in complex traits are usually below 100. A strong genetic contribution to overall risk of PSC is supported by λ_s values of approximately 100[29], as compared with values of 15-35 for Crohn's disease and 6-9 for UC[30].

Polymorphisms are genetic variants that have arisen from mutational events in DNA[31]. Conventionally, to be denominated a polymorphism, a mutant variant should occur at a frequency of > 0.01 in the general population. A particular nucleotide (or nucleotide sequence) at a polymorphism is defined as an allele. The combination of alleles on the two chromosomes is termed the genotype of the individual at that position. A distinct combination of two or more alleles of polymorphisms that occur together on the same chromosome is defined as a haplotype.

When a mutation arises in a chromosomal region, it does so on a background of particular DNA variants that are already present in the population, i.e. the mutation is linked to these surrounding alleles by the integrity of the DNA molecule. Over time, recombination tends to separate a mutant allele from the alleles of the surrounding DNA. At the population level, the positive association that remains between particular alleles at linked polymorphisms is called linkage disequilibrium (LD), meaning that these alleles occur more frequently together than would be expected from their population frequencies. Recombination ultimately leads to loss of LD unless there is a selective advantage of particular allele combinations.

The relationship between disease phenotype and three of the genetic concepts described (polymorphisms, alleles and haplotypes), is the subject of *genetic association studies*. That is, the aim of genetic epidemiology is to identify alleles (or in diploid terms, genotypes) of polymorphisms that are associated with an increase or decrease in risk of disease or a particular characteristic of a disease. The advantage of LD is that all polymorphisms in a genetic region do not have to be genotyped to detect an association. This is because the causative variant will reside on the same haplotypes as other polymorphisms and can be indirectly detected by typing for these. The disadvantage of LD is that it may be almost impossible to determine which of a series of alleles in LD on a haplotype that is actually the causative variant. Most of the genetic variation (> 99%) in the human genome is believed to be without any phenotypic consequence[32].

STATISTICAL CONSIDERATIONS

Because of the low prevalence, a major limiting factor for statistical power in studies of PSC susceptibility genes is sample size. Figure 2 illustrates the statistical power as a function of the effect size (odds ratio; OR) and allele

frequency of a genetic variant for studies performed in the largest PSC population in which studies have been performed so far (n = 365)[33]. Two issues require mentioning. First, very weak effects (OR ≈ 1.0-1.3) are likely to be missed, even for populations of this size. Second, rare variants of importance for PSC susceptibility (allele frequency < 0.01) are likely to be missed unless the OR of the variant is very high (or low; ORs < 1 were not plotted for clarity).

An important controversy regarding the prospects of mapping the genetic predisposition to complex diseases is not related to statistical power, but the possible complexity of allelic variation at a susceptibility locus. Supporters of the "common-disease/common-variant" hypothesis argue that common diseases arise due to polymorphisms that are common (i.e. allele frequency > 0.10[34]) in the background population. Supporters of the "multiple rare variants" hypothesis point to the complexity observed at susceptibility loci in monogenic disorders, where multiple rare alleles define a similar phenotype (e.g. the hundreds of disease causing alleles at the cystic fibrosis transmembrane-conductance regulator locus)[35]. Possibly, susceptibility genes in complex diseases that are defined by multiple rare variants cannot be identified using regular LD based approaches[36]. Although PSC is relatively rare, the main HLA haplotypes that confer risk are relatively common (e.g. the frequency of the PSC associated ancestral HLA haplotype 8.1 is > 0.10 in Scandinavia[37]).

The abundance of false positive genetic association studies (i.e. type I statistical errors) represents a problem of legitimacy for this type of study design[38]. Simply using a P-value < 0.05 as "evidence" to distinguish between a "positive" and "negative" finding in these studies can be questioned[39]. The problem is partly related to the many statistical tests performed in these studies. The so-called Bonferroni correction (multiplying P-values with the number of comparisons that have been performed) is the most widely accepted strategy to account for this problem.

The Bonferroni approach has limitations. Due to the many tests that are theoretically possible throughout the genome, it can be argued that conservative significance levels of 10^{-5} or even 10^{-8} should be used for all tests[38,40]. Achieving such significance levels would require patient collections simply not available for rare diseases like PSC. The most recent proposal is that so-called permutation testing (in Latin, "permutare" means "change completely") within a dataset is the preferable strategy to take account of multiple testing[41]. In permutation tests, case/control assignment is shuffled randomly using a computer and tests are run over and over again to count how often the permuted dataset achieves the effect observed in the correctly ordered dataset. If the permuted dataset achieves an effect equal to or stronger than that observed in the original dataset in 500 out of 10 000 analyses, this means that the probability of a type I error for a finding is 5%.

The problem of statistical significance in genetic association studies philosophically relates to the problem of causality for which criteria relevant to modern medicine were proposed by Sir Austen Bradford Hill in a classic essay in 1965[42]. These criteria point to factors in addition to the probability from statistical association tests (e.g. biological plausibility) that are required for a causal relationship to be established. This is also argued for in so-called Bayesian statistics, where the prior probability of a genetic variant to be associated (e.g. non-synonymous polymorphism in a gene which function is relevant to the disease phenotype), is accounted for when deciding on the posterior probability of whether or not a finding is valid[38]. In sum, circumstantial evidence (from functional studies or mouse models) is required to support findings if a genetic variant should be considered causative in terms of contributing to a disease phenotype[28], whatever the statistical evidence is available.

THE HLA COMPLEX AND GENETIC ASSOCIATIONS OBSERVED IN PSC

The HLA complex stretches across 7.6 million base pairs (bp) of DNA on the short arm of chromosome 6 and contains 252 expressed protein-coding genes, of which 28% are potentially related to immunological functions[43]. Throughout evolution of this genetic region[44], duplications have led to several gene clusters containing genes of similar function (Figure 3)[43]. HLA class I molecules (i.e. HLA-A, -B and -C) are expressed on all nucleated cells in the body and present intracellular/endogenous antigens to $CD8^+$ T-lymphocytes. HLA class I molecules also serve as ligands for inhibitory killer immunoglobulin-like receptors (KIRs) on natural killer (NK) cells and γδ T-lymphocytes[45,46]. HLA class II molecules are expressed on antigen presenting cells (e.g. macrophages and dendritic cells) and present extracellular/exogenous antigens to $CD4^+$ T-lymphocytes[45].

Sequence-based HLA-nomenclature was established in 1987[45]. The locus name is followed by an asterisk and two pairs of digits. The first pair of digits denominates the main type and is often similar to the serological type (e.g. DRB1*03 is the same as serological DR3, but DRB1*13 is only one of the DR6 alleles). The second pair of digits denominates the subtype (e.g. DRB1*0301 and DRB1*1301). Further definition is possible, since null alleles are suffixed by "N", and polymorphisms that do not alter the amino acid sequence of the peptide binding groove give rise to the fifth, sixth and seventh digits. In result, a complete sequence-based HLA allele name represents the haplotype of all alleles at all polymorphisms within the HLA gene at that chromosome.

LD between alleles at the HLA class I and II loci defines ancestral HLA haplotypes (AHs) and are named after which HLA-B allele they contain (e.g. the most common haplotype with HLA-B*08 is called AH8.1)[44]. Alleles of other genes are in LD with these ancestral haplotypes, and the co-occurrence of particular alleles across the entire HLA complex on one chromosome is called an extended HLA haplotype[47]. At the population level, the degree of conservation varies between different extended HLA haplotypes[48]. As examples of this phenomenon, an extended HLA haplotype with the HLA-B*08 and DRB1*0301 alleles (i.e. the AH8.1) is remarkably conserved in the Northern European population, whereas haplotypes carrying DRB1*04 alleles

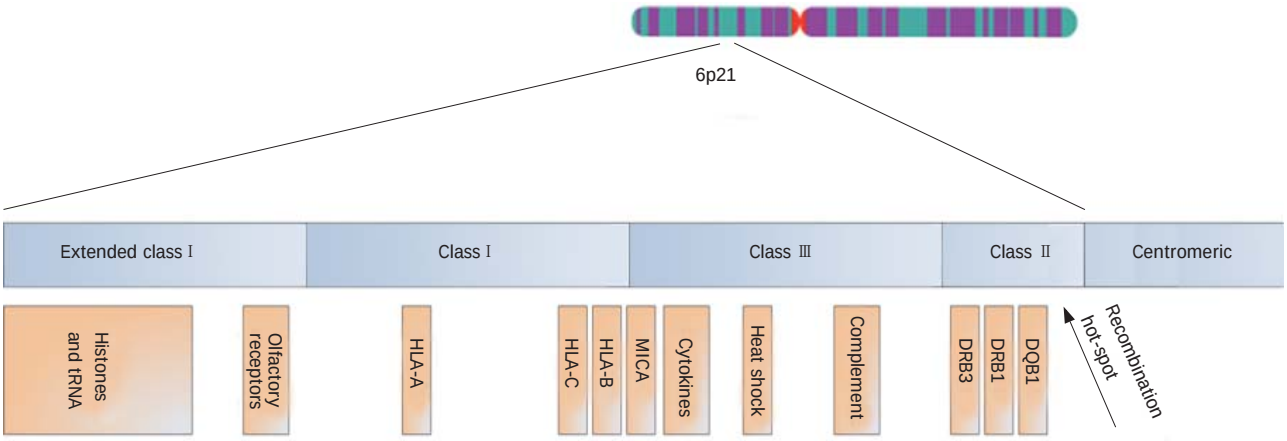

Figure 3 Schematic outline of the HLA complex on chromosome 6. Distances are arbitrary. By convention, the extended HLA complex stretches from the centromeric border of the HLA class Ⅱ loci (HLA-DP) to the telomeric limit of the histone gene cluster more than 4 million bp from HLA-A[43,120]. Centromeric to the HLA-DQ loci, a region with intense recombination can be found ("recombination hot-spot")[132].

are considerably less conserved and may not even qualify for the denomination "extended haplotypes"[49].

A HLA association in PSC was first identified for HLA-B8 (i.e. HLA-B*0801) and DR3 (i.e. DRB1*0301)[24,50]. Later studies have verified that PSC associations exist also for the other alleles of the AH8.1 (the HLA-A1 allele[51], the HLA-C7 allele[52], the major histocompatibility complex class I chain-related A (*MICA*) *008/5.1 allele[53,54], and the tumour necrosis factor alpha (*TNF*α) promoter -308 A allele[55,56]). This haplotype is associated with a wide range of autoimmune diseases[57,58]. A cross-European study (Norway, Sweden, Great Britain, Italy and Spain) concluded that a consistent, positive HLA class Ⅱ association in PSC probably exists also for a haplotype that carries the DR6 (i.e. DRB1*1301) allele[37]. In individuals negative for DR3 and DR6, an association with haplotypes that carry the DR2 (i.e. DRB1*1501) allele can be found. Negative associations with HLA class Ⅱ alleles have been reported for the DR4, DR7 and DR11 alleles[37,59,60], although primarily in populations of Northern European origin[56]. In Southern Europe, the picture is even more complex, since the DR4 allele seems to be consistent in LD with a predisposing variant in Italy[37,56], whereas a protective effect is noted in Spain[37].

Due to strong LD, an important question in HLA genetics is whether genetic associations are due to variation in the HLA class I or Ⅱ genes (meaning that they arise because the patients are able to present particular antigens to the T-cell receptor)[61], or due to variation in neighbouring genes[62]. There is some degree of amino acid sequence similarity between several of the PSC associated HLA class Ⅱ polypeptide variants[59,63]. However, no consistency has been found regarding these similarities[59]. The proposal of leucine at position 38 of the DRβ polypeptide as a critical determinant for PSC susceptibility relies heavily on the strong DRB3*0101 association in Northern European populations[63]. An early suggestion that a common denominator between haplotypes with the DRB1*0301 and DRB1*1301 alleles could be the DRB3*0101 allele (serologically DRw52a) was later withdrawn[64,65]. Another study found that the DRB1*1301-DRB3*0202 haplotype association is as strong as the DRB1*1301-DRB3*0101 association[37]. Taken together, the most interesting proposal of a single amino acid position in defining risk of PSC may rather relate to a protective effect in carriers of proline at position 55 of the DQβ polypeptide, which is common for DQ3 alleles known to be in LD with the protective DR4, DR7 and DR11 alleles[59]. However, no consistent risk allele is defined by this position[59], and to what extent the HLA class Ⅱ molecules are of primary importance in the PSC pathogenesis should probably not be concluded based on present evidence.

The PSC-associated MICA*008/5.1 allele has been proposed as a common denominator between the PSC-associated A*01-C*07-B*08-DRB1*0301-DQB1*0201 and A*03-C*07-B*07-DRB1*1501-DQB1*0602 haplotypes[53,59]. MICA functions as a ligand for the activating NKG2D receptor on NK cells[66]. It was recently recognised that the two risk haplotypes in question share alleles not only at *MICA*, but also at the neighbouring *HLA-B* and *-C* loci, when these are defined according to the KIR binding properties of the HLA class I molecules[67]. The PSC-associated *HLA-B* and *-C* KIR ligand genotypes may result in decreased inhibition of NK cells and several subsets of T-lymphocytes that express KIRs[46,68]. Such combinations of KIR and HLA class I ligand variants have been shown to increase susceptibility to other autoimmune diseases[46]. How the PSC-associated MICA*008/5.1 allele may cause disease is not known. This allele is also associated with an increased risk of other autoimmune conditions[69,70], and may thus also result in an increased activity of cells expressing the NKG2D receptor, acting in synergy with the loss of inhibition resulting from the PSC associated HLA class I ligand genotypes. The fact that the MICA 5.1 allele was recently shown to confer protection against cholangiocarcinoma is in line with an activating effect[71]. Some studies report an increased frequency of NK cells in the portal infiltrate of patients with PSC when compared with other liver diseases[72,73], and also in the intestinal mucosa of patients with PSC without IBD compared with IBD patients without liver

Table 1 Candidate gene studies performed in PSC

Gene	Chromosome	N (PSC)	Primary finding	Reference	Replication finding	Reference
IL-1	2q	40	Negative	[76]	Negative	[77]
IL-10	1q	96	Negative	[77]	Negative	[55]
MMP1	11q	165	Negative	[78]	NA	-
MMP3	11q	111	Positive	[79]	Negative	[78]
CCR5	3p	71	Positive	[80]	Negative	[33]
ICAM-1	19p	104	Positive	[81]	Negative	[82]
CFTR	7q	29	Negative	[83]	Negative	[84,85]
MDR3	7q	37	Negative	[86]	Negative	[87]
BSEP	2q	37	Negative	[86]	NA	-
AIRE	21q	60	Negative	[88]	NA	-
NRAMP1	2q	40	Negative	[89]	NA	-
CTLA4	2q	144	Negative	[90]	NA	-
FOXP3	X	195	Negative	[91]	NA	-

Interleukin-1 and -10 (IL-1 and -10), MMP1 and 3 (matrix metalloproteinase 1 and 3), CCR5 (chemokine receptor 5), ICAM-1 (intercellular adhesion molecule 1), CFTR (cystic fibrosis transmembrane conductance regulator), MDR3 (multidrug resistance gene 3), BSEP (bile salt export pump), AIRE (autoimmune regulator), NRAMP1 (natural resistance-associated macrophage protein 1), CTLA4 (cytotoxic T-lymphocyte-associated protein 4), FOXP3 (forkhead box P3). NA: Not available.

disease[74]. Taken together with the genetic findings in this region of the HLA complex (Figure 3), further studies on the role of these cells in PSC seem warranted.

In sum, the HLA association in PSC is likely to be complex. Multiple risk variants may exist[25], some of which may be associated not only with PSC, but autoimmunity in general.

PSC ASSOCIATIONS WITH POLYMORPHISMS IN GENES OUTSIDE THE HLA COMPLEX

Summarising the published genetic association studies in PSC, it seems proven beyond doubt that one or more genetic variants located within the HLA complex are important. The true identities of these variants, as discussed above, are not known. The situation is even less clear with regard to other susceptibility loci. Given the large number of protein coding genes in the human genome (25-35 000)[32], selecting candidate genes for association studies is an extremely difficult task. According to strict criteria for what may be denominated a susceptibility gene in complex diseases (consistent statistical evidence, functional consequence of identified mutation, relevant tissue expression, *etc.*)[28], no such gene exists for PSC. A summary of studies performed is given in Table 1. So far, most attention has been given to genes known to be of importance in other autoimmune diseases. The association between PSC and IBD has also inspired some of the studies, as well as the observation of PSC-like changes in cystic fibrosis[75].

Two of the negative findings are of particular interest and will be discussed in greater detail. First, studies in limited populations ($n < 50$) have pointed to a non-significant increase of particular multidrug resistance gene 3 (*MDR3*) variants among PSC patients as compared with healthy controls[86,87]. Knock-out mice for this phospholipid transporter gene (called *mdr2* in mice) spontaneously develop hepatic lesions resembling PSC[92], possibly due to loss of protection of the biliary epithelium from toxic bile acids. Second, it cannot be formally ruled out that the 32 bp deletion of the chemokine receptor 5 (*CCR5*) gene and the E/E genotype of the K469E SNP in the intercellular adhesion molecule 1 (*ICAM-1*) gene may confer population specific effects[80,81,93]. Both genes are plausible candidate genes in PSC. The CCR5 may be involved in the recruitment of intestinally activated lymphocytes *via* portal expression of CCR5 ligands (e.g. the macrophage inflammatory protein-1α and β), and ICAM-1 may play a similar role in recruiting leukocytes to an inflamed liver by interacting with the β2-integrin ligand. The negative findings in the replication series referred to in Table 1 state it unlikely that genetic variants of these receptors are of primary importance in the pathogenesis of PSC. The receptors may, however, still be involved in the disease process along with other CCRs and adhesion molecules [e.g. CCR9 and the mucosal addressin cell adhesion molecule 1 (MAdCAM-1)[94,95]].

GENETIC ASSOCIATIONS WITH CLINICAL SUBSETS OF PSC PATIENTS

The most prominent features of PSC along with the biliary changes are inflammatory bowel disease, cholangiocarcinoma and other autoimmune diseases (Figure 1).

The increased frequency of autoimmune diseases among patients with PSC is possibly due to the increased frequency of the AH8.1 among the patients[58,96]. Similarly, an increased frequency of IBD risk alleles among patients with PSC could contribute to the co-occurrence of these two phenotypes. Several IBD susceptibility genes have been identified during the last 6 years through the application of genome-wide linkage screens and subsequent fine-mapping approaches[26]. To determine if the high frequency of IBD among patients with PSC could be due to genetic risk factors shared with IBD in general, we recently genotyped key polymorphisms of known IBD susceptibility genes in a large cohort of Scandinavian PSC patients[97]. The following genes were studied: caspase activating recruitment domain 15 (*CARD15*), toll-like receptor 4 (*TLR-4*), caspase activating recruitment domain 4 (*CARD4*), solute carrier family 22, member 4 and 5 (*SLC22A4* and *SLC22A5*), Drosophila discs large homolog 5 (*DLG5*) and multidrug resistance gene 1 (*MDR1*)[26,98]. No significant PSC associations were detected for any of the investigated polymorphisms[97]. These negative findings add to notions that the IBD phenotype in PSC may be a "third" IBD phenotype[99], possibly distinct from UC and Crohn's disease not only in clinical presentation, but also with regard to genetic susceptibility.

It is of interest to know whether genetic associations detected in PSC may be of particular importance for the IBD phenotype among the PSC patients or patients with IBD in general. In a recent study of HLA alleles in

PSC and UC patients of the same ethnicity[100], the only parallel association detected was a protective effect of the DRB1*0404 allele, more pronounced among the PSC patients than among the patients with UC without liver disease. No association with any of the main PSC risk alleles (DRB1*0301, DRB1*1301 or DRB1*1501) was found among the regular UC patients. Interestingly, a non-significant trend towards a higher frequency of the DRB1*1501 allele was noted among the patients with PSC and concurrent IBD compared with PSC patients without IBD, and the possibility should be held open that this HLA haplotype may harbour genetic variants of particular importance for the IBD phenotype in PSC. A similar notion can be made with regard to the MMP3 5A allele association detected by Satsangi *et al*[79]. Although the replication study by Wiencke *et al*[78] failed to confirm an overall association with PSC susceptibility, a significant association was evident when PSC patients with UC were compared with UC patients without liver disease.

The study by Wiencke *et al*[78] also detected a possible association between cholangiocarcinoma and the MMP1 1G allele. Although the number of patients with cholangiocarcinoma in this series was too small for conclusive statistics to be performed (n = 15), the 100% occurrence of this allele among the cholangiocarcinoma patients warrants future replication attempts in other study populations. Recently, a highly significant association between polymorphisms in the *NKG2D* gene and cholangiocarcinoma in PSC was detected[71]. Previous studies have highlighted the importance of this activating NK cell receptor in protection against other cancer types[66]. Persistent exposure to effector molecules of inflammatory pathways (e.g. IL-6[101]), along with chronic cholestasis[102], is probably important for the malignant transformation of cholangiocytes. The study by Melum *et al*[33] points to the possible role of NK cell activity in protection against neoplastic cells. Polymorphisms of the *NKG2D* gene along with other parameters may also prove important in identifying PSC patients at a particular low risk of developing cholangiocarcinoma.

MODIFIER GENES IN PSC

There is an increasing interest in so-called "modifier genes" in complex diseases (as compared with "susceptibility genes"), initiated by the recognition of the influence of such genes on disease expression (e.g. severity) in monogenic disorders like cystic fibrosis and haemochromatosis[103-105]. Modifier genes may point to biochemical and physiological systems of relevance to prognosis and are therefore of great clinical interest. Although PSC should be considered a progressive condition culminating in death or liver transplantation in most cases[106], the clinical course for each individual patient varies considerably[107,108]. In terms of disease course, indicators of PSC severity (e.g. portal hypertension and need for liver transplantation) are more likely to represent a particular disease stage than to serve as valid measures of disease progression. The most precise strategy for performing enquiries on effects from genotypes on disease course in PSC is thus to compare absolute survival time (defined as time from diagnosis until death or liver transplantation) using Kaplan-Meyer analyses, or calculating the relative risk for death and/or liver transplantation from Cox regressions[109,110].

We have recently observed that genetic variants of the steroid and xenobiotic receptor (SXR) are associated with a more aggressive disease course in PSC[110]. The SXR is a ligand-dependent transcription factor known to mediate protection against bile acid-induced liver injury in cholestatic animal models[111,112]. In this perspective, our data may suggest that the activity of bile acid detoxification systems could be of importance for disease progression in PSC. Interestingly, the SXR ligand rifampicin has been used in the treatment of cholestatic pruritus[113], and it has also been shown that ursodeoxycholic acid is able to activate SXR in human hepatocytes[114]. However, the SXR may also influence inflammatory pathways *via* the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB)[115], as well as liver fibrogenesis and thus cirrhosis *via* direct effects on hepatic stellate cells and Kuppfer cells[116]. Further studies are needed to clarify the functional consequences of various polymorphisms of the *SXR* gene in patients with PSC.

The SXR variants associated with death or liver transplantation in our study were not associated with PSC susceptibility[110]. However, also for some of the disease-associated variants in the HLA complex, modifier effects have been observed. The first notion was made by Gow *et al*[117] who described an unusually aggressive disease progression in four patients carrying the DR4 allele. Later, Boberg *et al*[109] found that DR4 positive patients have an increased risk of cholangiocarcinoma, but do formally not experience an accelerated disease progression. In this study, an increased risk of death or liver transplantation was observed in patients heterozygous for the DR3-DQ2 haplotype. As long as the causative variants along the HLA haplotypes in question have not been identified, one can only hypothesize upon a biological explanation for these observations. Given the complexity of the HLA associations in PSC, it is even possible that other variants within this region may be important for disease progression than those primarily important for disease susceptibility. However, for the same reasons it has been difficult to pinpoint susceptibility genes in this region (strong LD, multiple genes of immunological relevance, *etc*.), such modifier genes may prove hard to identify conclusively.

FUTURE STUDIES AND CONCLUDING REMARKS

Although several important findings have been made during the past 25 years since the first genetic association study in PSC was performed[24], PSC remains an enigmatic disease and future studies are warranted. With an ever increasing availability of methods for efficient genotyping of polymorphisms[118], a critical limitation for such studies in PSC is the availability of well-characterised patient materials. Collaborative efforts will be necessary to achieve patient collections required for detecting the modest effects (Figure 2), as well as for replicating results of

uncertain validity[33]. Such collaborations are now being undertaken in other diseases[119], and have successfully aided in clarifying genetic associations found in PSC[37].

In terms of future research strategies, several proposals can be made. First, dissection of the widely replicated HLA-associated susceptibility to PSC should be considered a priority. Detailed maps of genetic markers in this region are now available[120]. It is anticipated that the systematic application of such marker maps in populations of an appropriate size may lead to the identification of true, disease causing variants in this difficult region[62].

Second, some biological pathways are pointed to by existing findings (e.g. the possible importance of bile acid homeostasis in influencing disease progression), and further candidate gene studies of critical components of these systems may identify additional risk factors. There is increasing awareness of the importance of interaction between polymorphisms in functionally related genes in complex diseases, i.e. epistasis[121,122]. In some cases, epistatic considerations have proven necessary for the detection of effects from genetic variation on a phenotype of interest[123,124]. These observations have implications for study design in future candidate gene studies in PSC. Polymorphisms not only in single genes, but in relevant panels of several genes encoding proteins with closely related functions, should be investigated.

Finally, two recent advances in the genetic research field now make genome-wide studies feasible also for case-control materials. First, the human haplotype map project (HAPMAP) was recently completed[125]. In the project, 3.9 million SNPs have been genotyped in families of three different ethnicities (at the time of writing). Results from the project enable researchers worldwide to efficiently select SNPs throughout the genome that are prone to cover genetic variation of interest to a project[126,127]. Second, although costs are high, genotyping technology now allows for the typing of 100 000's of SNPs simultaneously in the same DNA sample[118]. Emerging reports provide proof-of-concept for genome-wide case-control studies[128,129]. However, there are still statistical problems to be solved regarding the many tests performed and risk of false positive results[130]. As evident from Figure 2, only strong effects may be detectable, and prospects may not yet justify the costs. However, sooner or later genome-wide studies seem warranted, also in PSC. Possibly, PSC susceptibility genes will be identified that would otherwise never have been included in hypothesis-driven candidate gene studies of the type performed so far[131].

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CLINICAL RESEARCH

Ribavirin and IFN-α combination therapy induces CD4+ T-cell proliferation and Th1 cytokine secretion in patients with chronic hepatitis B

Fen-Yu Ren, Hai Jin, Xi-Xu Piao, Feng-Shun Piao

Fen-Yu Ren, Hai Jin, Xi-Xu Piao, Feng-Shun Piao, Department of Gastroenterology and Hepatology, Yanbian University Hospital, Yanji 133000, Jilin Province, China
Correspondence to: Fen-Yu Ren, MD, PhD, Department of Gastroenterology and Hepatology, Yanbian University Hospital, Yanji 133000, Jilin Province, China. fenyu65@yahoo.com.cn
Telephone: +86-433-2660061 Fax: +86-433-2513610
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Abstract

AIM: To investigate the anti-viral mechanism of combination therapy of interferon (IFN)-α and ribavirin in patients with chronic hepatitis B.

METHODS: Twenty patients were assigned to receive either IFN-α plus ribavirin (group A, *n* = 14) or no treatment as a control (group B, *n* = 6). Patients were analyzed for T-cell proliferative responses specific for hepatitis B virus (HBV)-antigen and cytokine production by peripheral blood mononuclear cells (PBMCs).

RESULTS: Combination therapy induced HBV-antigen specific CD4+ T-cell proliferative responses in four patients (28.6%). Production of high levels of HBV-specific IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-12 by PBMCs was found in five patients (35.7%), who showed significantly lower HBV DNA levels in serum at 12 mo after treatment ended (P = 0.038) and at 24 mo of follow-up (P = 0.004) than those without high levels of cytokine production.

CONCLUSION: HBV-antigen specific CD4+ T cells may directly control HBV replication and secretion of anti-viral T helper 1 (Th1) cytokines by PBMCs during combination therapy of chronic hepatitis B with ribavirin and IFN-α.

Key words: Hepatitis B; Interferon-alpha; Ribavirin; CD4+ T cells; Th1

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INTRODUCTION

More than 400 million people worldwide have chronic hepatitis B virus (HBV) infections[1]. Chronically infected patients with active liver disease have a high risk of developing cirrhosis and hepatocellular carcinoma[2]. However, therapeutic options against HBV still present a major clinical challenge. The goal of treatment is HBV DNA suppression, normalization of alanine aminotransferase (ALT) levels and reduction in liver necroinflammation. Currently available therapies against HBV are mainly interferon (IFN)-α and nucleoside analogs, which are well tolerated and induce a decrease in serum HBV DNA levels and normalization of serum ALT levels. However, the efficacy of IFN-α[3,4] or nucleoside analogs for treatment of hepatitis B varies in different clinical situations[5-12]. IFN-α shows seroconversion from hepatitis B e antigen (HBeAg) to antibody to HBeAg (anti-HBe), concomitant with HBV DNA negativity in just one-third of patients treated, and is both costly and induces adverse effects[3]. It has been well established that IFN-α has potent antiviral activity against DNA and RNA viruses, and that it also acts as an immunomodulatory agent[13]. Some reports have suggested that ribavirin shows antiviral and immune effects against various infections[14], including hepatitis B and C. Both drugs have the capacity to modulate systemic as well as virus-specific T-cell responses, along with the potential to shift the profile of cytokine secretion[15,16].

Recent reports have suggested that combination therapy with IFN-α plus ribavirin for chronic hepatitis B significantly reduces viremia[17,18] and induces lasting CD4+ T-cell proliferation and Th1 cytokine release at the site of infection, which may lead to sustained HBV eradication[18]. These preliminary data in anti-HBe-positive patients refractory to IFN-α treatment appear to be promising[18]. Thus, in the present study, we investigated the mechanism involved in the control of HBV replication, utilizing combination therapy with ribavirin and IFN-α.

MATERIALS AND METHODS

Patients

Twenty patients with chronic hepatitis B (14 men and 6 women; mean age 42 years), positive for both anti-HBe and HBV DNA in the serum, and who had failed previous IFN-α treatment were enrolled in this prospective trial. None had human immunodeficiency virus, hepatitis

C virus, or hepatitis D virus infections, hepatocellular carcinoma, or had received nucleoside analogs. Six healthy controls were also analyzed (mean age 38 years). Table 1 shows patient characteristics at enrollment. This study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by institutional ethics committee. Informed consent was obtained from all patients prior to inclusion.

Therapeutic and analytic schedule

The patients were divided into two groups: group A (n = 14) for combination therapy with IFN-α plus ribavirin, and group B (n = 6) for untreated controls. Patients in group A received 5 million U IFN-α2b three times a week for 12 mo, plus ribavirin (1000 mg/d) orally for 12 mo[17]. Patients were followed for 12 mo after the end of treatment. Blood chemistry, blood cell counts, and HBV DNA were measured at the beginning of treatment, and then every 1-2 mo during treatment and follow-up periods. HBeAg and anti-HBe were measured at the beginning of treatment, and then at 6-mo intervals. Blood samples for immunological analysis were collected before therapy and at 3, 6, 9, 12, 18 and 24 mo after the commencement of therapy. Serum HBV DNA was quantified, using transcription-mediated amplification and a hybridization assay. The concentration of HBV DNA in the samples was expressed as the logarithm of genome equivalents per milliliter (LGE/mL). Biochemical and hematologic parameters were measured by standard methods. All patients completed treatment and follow-up.

Cell preparation

The methods of cell preparation used here were nearly identical to those of Ren *et al*[19] in their report focusing on therapeutic vaccination against chronic hepatitis B. Peripheral blood mononuclear cells (PBMCs) from 20 patients were separated from heparinized blood by density-gradient centrifugation with lymphoprep (Nycomed Pharma AS, Oslo, Norway). B cells were removed from PBMCs by negative depletion, by incubating the cells with mouse anti-$CD19^+$ antibodies coated on magnetic beads (Danal, Olso, Norway). CD4+ or CD8+ T cells were then removed from the resultant T cells in the same manner, using mouse anti-human CD4+ or CD8+ antibodies coated on magnetic beads (Danal), respectively. CD4+ cells were also blocked with CD4+ antibodies (Caltag Laboratories, Burlingame, CA, USA). The purity of the T-cell subpopulation was monitored by immunolabeling with anti-$CD3^+$ antibodies (Becton Dickinson, San Jose, CA, USA). Flow cytometry revealed a > 95% purified T-cell subpopulation. PBMC or lymphocyte subsets were resuspended with 2 mmol/L l-glutamine, 10 mmol/L HEPES, 100 kU/L penicillin, 100 mg/L streptomycin, and 50 mL/L human AB serum (complete medium).

Proliferation assay

The proliferation assay used in this study was nearly identical to that of Ren *et al*[19]. T cells (1.5×10^6 cell/L in 0.2 mL complete medium) were cultured in triplicate wells of 96-well round-bottom microplates with medium alone, were stimulated with 10 mg/L phytohemagglutinin (PHA: Sigma) and 3 mg/L HBV antigens: HBsAg protein, synthetic entire preS1, HBeAg, and hepatitis B core antigen (HbcAg) (Virostat, Portland, ME, USA). After 4 d of culture at 37℃ in an atmosphere of 50 mL/L CO_2 in air, the cells were labeled for 18 h with 37 kBq of [^{3}H]-thymidine (Amersham, Little Chalfont, UK). DNA-incorporated radioactivity was measured by scintillation counting. Data were expressed as the stimulation index (SI), calculated as the ratio of the mean cpm of triplicate cultures obtained in the presence of antigen to cpm obtained without antigen. SI > 3 was considered significant. The proliferative responses were not tested with PBMCs from patients in control group B.

Table 1 Clinical characteristics of patients at enrollment

Patient No.	Age (yr)	Gender	ALT (nkat/L)	HBV DNA (10^3 LGE/L)	Type of response (end of treatment)
Group A					
1	48	M	89	6.8	Responder
2	51	M	158	7.3	Responder
3	44	M	86	8.3	Responder
4	41	M	464	8.0	Responder
5	39	M	122	4.8	Responder
6	67	M	142	8.1	Non-responder
7	52	M	140	8.3	Non-responder
8	26	M	69	4.8	Non-responder
9	52	M	46	7.6	Non-responder
10	33	M	39	5.4	Non-responder
11	24	F	458	8.7	Non-responder
12	27	F	51	8.5	Non-responder
13	52	F	80	7.4	Non-responder
14	37	F	57	6.8	Non-responder
Group B					
15	42	F	43	7.2	No treatment
16	33	F	19	8.1	No treatment
17	51	M	138	3.9	No treatment
18	31	M	57	7.1	No treatment
19	29	M	79	7.1	No treatment
20	44	M	46	7.2	No treatment

P > 0.05, Group A *vs* Group B.

Cytokine assay

The cytokine assay used in this study was nearly identical to that of Ren *et al*[19]. PBMCs (1.5×10^6 cell/L in 0.2 mL complete medium) were cultured in triplicate wells of 96-well round-bottom microplates with medium alone, and stimulated with 10 mg/L PHA or with 3 mg/L HBsAg, HBeAg, HBcAg or preS1. After 3 d of culture at 37℃ in an atmosphere of 50 mL/L CO_2 in air, culture supernatants were collected. Concentrations of IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-10 and IL-12 p70 were determined using commercial ELISA kits (Genzyme, Cambridge, MA, USA). Production levels after antigen stimulation were expressed as the ratio of the mean cytokine concentration of triplicate cultures obtained in the presence of antigen to that obtained without antigen.

Statistical analysis

Results are expressed as mean ± SD. Differences in proportions were tested by the χ^2 test. Mean quantitative values were compared using the Mann-Whitney U test. All reported P values were two-tailed and $P \leqslant 0.05$ was considered significant.

Table 2 SI of T cells against HBV antigens from combination therapy patients

Patient No.	12 mo					24 mo				
	PHA	HBsAg	preS1	HBeAg	HBcAg	PHA	HBsAg	preS1	HBeAg	HBcAg
1	38.1[1]	3.5[1]	3.2[1]	3.9[1]	4.2[1]	8.2[1]	4.1[1]	3.5[1]	4.1[1]	4.8[1]
2	20.3[1]	3.4[1]	4.0[1]	4.1[1]	4.4[1]	7.7[1]	3.2[1]	3.0	4.4[1]	4.7[1]
3	19.5[1]	3.2[1]	3.9[1]	4.8[1]	4.1[1]	4.9[1]	3.5[1]	3.7[1]	4.7[1]	5.1[1]
4	32.3[1]	4.3[1]	7.8[1]	7.2[1]	7.9[1]	9.6[1]	4.0[1]	6.9[1]	8.1[1]	8.8[1]
5	15.5[1]	1.9	2.3	2.0	1.7	8.4[1]	3.5[1]	3.5[1]	4.3[1]	4.4[1]
6	12.8[1]	0.4	1.2	1.5	1.4	4.9[1]	1.1	1.3	1.5	1.2
7	50.2[1]	2.1	1.9	2.0	2.0	38.8[1]	1.7	1.8	1.6	1.6
8	4.9[1]	1.8	2.1	1.7	1.9	NT	NT	NT	NT	NT
9	66.3[1]	2.0	1.5	1.8	1.6	12.0[1]	1.5	1.2	1.5	1.3
10	56.6[1]	2.3	2.3	2.0	2.1	18.3[1]	2.0	2.1	1.8	1.9
11	23.8[1]	1.1	1.0	1.1	1.2	8.7[1]	1.0	0.8	1.2	1.3
12	19.2[1]	1.4	1.2	1.4	1.3	4.6[1]	1.1	1.5	1.1	1.2
13	42.0[1]	1.8	1.5	1.9	1.7	6.9[1]	1.5	1.3	1.4	1.5
14	21.8[1]	2.2	2.4	2.2	2.6	4.9[1]	2.0	2.3	1.9	2.0

[1]SI > 3 correspond to significant proliferative responses. NT, not tested; PHA (10 mg/L); HBV antigen (3 mg/L).

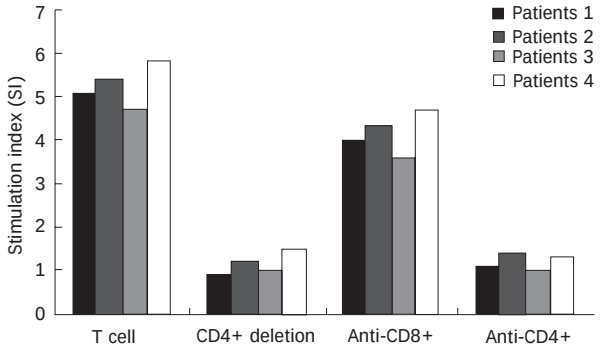

Figure 1 Abrogation of antigen-specific T-cell proliferative responses from patients 1-4 at 12 mo ([b]$P < 0.01$ *vs* T cell and Anti CD8+).

RESULTS

Clinical outcome

HBV DNA levels decreased, with a significant reduction at 9 mo and thereafter, as compared to those at baseline, in the combination therapy patients. Serum HBV DNA levels were also significantly lower in the combination therapy patients than in the controls at 12 and 24 mo. At 12 mo, four patients in group A (28.6%) (patients 1-4) had undetectable HBV DNA levels, and also showed sustained normalization of ALT; they were thus considered sustained responders, as previously reported[17,18]. The remaining 10 patients at 12 mo had detectable HBV DNA and elevated ALT levels.

Induction of T-cell proliferation response to HBV antigens

Proliferative responses of T cells during combination therapy and the follow-up period are summarized in Table 2. Four patients (1-4) showed significant proliferative responses at 12 mo, and these responses were sustained until 24 mo (the end of follow-up). These proliferative responses were always specific to both HBV antigens. Thus, this combination therapy was found to have induced proliferative T cell responses specific to the antigen contained in these four patients (28.6%). Patient 5 also showed a strong proliferative response at 24 mo. However, whether the combination therapy induced this response is unclear because it occurred at 12 mo after completion of therapy.

T-cell proliferative responses were also examined for patients 1-4 after incubation with anti-CD4+ antibodies or removing CD4+ or CD8+ cells. Depletion of CD8+ cells did not clearly inhibit the proliferative responses, while depletion of CD4+ cells or blocking with anti-CD4+ antibodies completely abrogated the proliferative responses of the patients (Figure 1).

HBV-specific cytokine production in PBMCs

HBV-specific cytokine production levels of PBMCs are shown in Figure 2. Cytokine production showed a Th1-like pattern characterized by secretion of IFN-γ, TNF-α, and IL-12 in the absence of IL-4 and IL-10 in five patients (1-5, defined as responders). Patients 1-4 exhibited remarkable increases in IFN-α, TNF-α, and IL-12 production at 3, 6 or 9 mo, as compared to patient 5 who showed mild, but not significant, proliferative responses (Table 2). These responses were sustained until the end of the observation period. Production of Th1 (IFN-γ, TNF-α, and IL-12) and Th2 (IL-4 and IL-10) cytokines did not increase, or remained unchanged in the patients who received other combination therapy (patients 6-14, defined as non-responders).

The mean serum HBV DNA level was lower in responders than in non-responders at 12 mo (4.3 ± 1.1 *vs* 6.0 ± 1.1, $P = 0.038$) and 24 mo (3.8 ± 0 *vs* 5.7 ± 1.2, $P = 0.004$). It is noteworthy that HBV DNA fell to under the detection limit at 24 mo in all responders. The decrease in serum HBV DNA level was almost coincident with the increase in IFN-γ production by HBV antigen-specific T cells, but was not preceded by any increase in serum ALT levels in four responders (patients 2-5).

DISCUSSION

In the present study, a significant decrease was found in serum HBV DNA levels at 9 mo and thereafter, along with significantly lower levels of HBV DNA in the combination therapy patients than in the controls at 12 and 24 mo. IFN-α plus ribavirin therapy appeared to inhibit

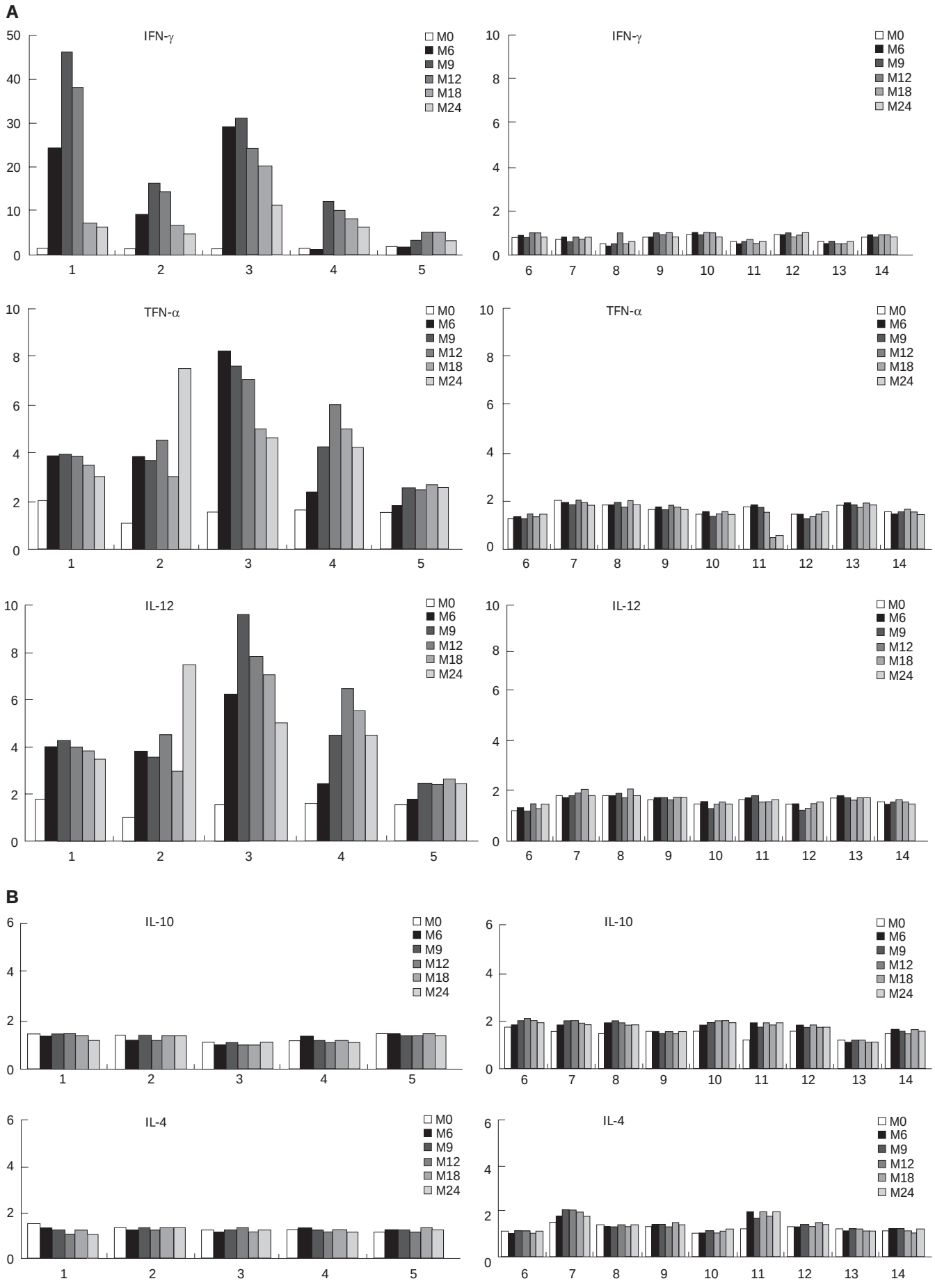

Figure 2 Cytokine production levels in PBMCs in combination therapy patients. The vertical axis represents the ratio of the mean cytokine concentration of triplicate cultures obtained in the presence of antigen to that obtained without antigen. The numbers in the horizontal lines represent the patients. **A:** Th1 cytokine production in PBMCs in combination therapy patients; **B:** Th2 cytokine production in PBMCs in combination therapy patients.

HBV replication in some patients, since serum HBV DNA levels were significantly lower at 12 and 24 mo in responders who showed HBV-antigen-specific IFN-γ, TNF-α and IL-12 production *in vitro* in PBMCs that was augmented in responders. The production of HBV-antigen-specific Th1 (IFN-γ, TNF-α, and IL-12) and Th2 (IL-4 and IL-10) cytokines did not increase, or remained unchanged in non-responders. Cytokine production showed a Th1-like pattern, as well as induction of PBMCs, and was consistent with the results of Rico *et al*[18].

CD4+ T cells are necessary for the maintenance of the effector functions of CD8+ T cells during chronic viral infection[20]. Activated CD8+ cytotoxic T cells can kill virus-infected cells by utilizing both perforin-dependent and Fas-mediated cytotoxic mechanisms[21]. CD8+ T cells can also secrete anti-viral cytokines such as IFN-γ and TNF-α[22]. The HBV-antigen-specific T-cell reactivity observed in our study may be relevant for the outcome of the infection, because it may be crucial to provide help to CD8+ cytotoxic T-cell responses to lyse and clear HBV-infected cells[23-27]. Nevertheless, eradication of HBV may be accomplished by other cells non-cytolytically by transcription and replication of HBV[28,29]. Based on our results, it is not possible to establish the mechanism contributing to HBV clearance (in this study we did not investigate CD8+ cytotoxic T cells in the cytotoxic assay). However, the decrease in serum HBV DNA levels was almost coincident with the increase of IFN-γ production by antigen-specific CD4+ T cells and was not preceded by an increase in serum ALT levels (as represented by patient 2, data not shown). These results suggest that cytotoxic T cells are unlikely to contribute to the control of HBV replication in combination therapy with ribavirin and IFN-α; results that are supported by those of Rico *et al*[18]. CD4+ T cells appear to directly participate in the anti-viral response (by producing anti-viral cytokines) rather than indirectly (by helping cytotoxic T cells) in this combination therapy of ribavirin and IFN-α. A role for Th1 cells in controlling viral infection is supported by experiments showing that they can clear influenza[30,31] and vaccinia virus[32] infections in a cytotoxic-T-lymphocyte-independent manner. Further, a direct, cytokine-dependent anti-viral role for CD4+ T cells, which produce Th1 cytokines (Th1 cells), has been shown in HBV transgenic mice[33,34]. These reports support our concept that the increased production of anti-viral cytokines by PBMCs plays a crucial role in the control of HBV replication in combination therapy with IFN-α and ribavirin. This combination therapy for chronic hepatitis B not only significantly reduced viremia levels but also induced lasting CD4+ T-cell proliferation and Th1 cytokine release at the site of infection, which may have led to sustained HBV eradication, as suggested by Rico *et al*[18]. Further studies will be needed to ascertain whether the anti-viral mechanism of combination therapy is by a route different from the one normally employed.

In conclusion, the present study indicated that combination therapy with ribavirin and IFN-α for anti-HBe-positive patients significantly reduces viremia, and induces CD4+ T-cell proliferation and Th1 cytokine secretion in patients with chronic hepatitis B.

COMMENTS

Background

Some recent reports have suggested that ribavirin shows antiviral and immune effects against various infectious diseases, including hepatitis B and C. It is suggested that combination therapy with IFN-α plus ribavirin for chronic hepatitis B significantly reduces viremia; however, the mechanisms involved remain unclear.

Research frontiers

Previous studies have suggested that combination therapy with IFN-α plus ribavirin for chronic hepatitis B significantly reduces viremia; however, the mechanism is unclear. In the present study, we investigated the anti-viral mechanism of combination therapy with IFN-α and ribavirin against chronic hepatitis B by analyzing T-cell proliferative responses in patients and determining Th1 cytokine levels.

Innovations and breakthroughs

In this study, we analyzed HBV-specific CD4+ T-cell proliferative responses and determined Th1 cytokine levels in PBMCs. Our results indicated that combination therapy of patients with chronic hepatitis B with ribavirin and IFN-α significantly reduced viremia, and induced CD4+ T-cell proliferation and Th1 cytokine secretion.

Applications

This study indicates that combination therapy with ribavirin and IFN-α for chronic hepatitis B significantly reduces viremia; thus, this combination may represent an alternative treatment option to achieve sustained eradication of HBV in patients with chronic hepatitis B refractory to IFN-α treatment.

Peer review

This is an interesting report of combination therapy with IFN-α plus ribavirin against chronic hepatitis B. The results suggest that HBV-antigen-specific CD4+ T cells may directly control HBV replication and secretion of anti-viral Th1 cytokines by PBMCs, utilizing combination therapy with ribavirin and IFN-α against chronic hepatitis B.

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CLINICAL RESEARCH

Influence of a nucleotide oligomerization domain 1 (*NOD1*) polymorphism and *NOD2* mutant alleles on Crohn's disease phenotype

Elisabet Cantó, Elena Ricart, David Busquets, David Monfort, Esther García-Planella, Dolors González, Joaquim Balanzó, José L Rodríguez-Sánchez, Sílvia Vidal

Elisabet Cantó, José L Rodríguez-Sánchez, Sílvia Vidal, Department of Immunology, Sant Pau Hospital & Research Inst. Sant Pau Hospital, Barcelona 08025, Spain
Elena Ricart, Department of Gastroenterology, CIBER-EHD. Clinic Hospital, Barcelona-08025, Spain
David Busquets, David Monfort, Esther García-Planella, Dolors González, Joaquim Balanzó, Department of Digestive Pathology, Sant Pau Hospital, Barcelona 08025, Spain
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Correspondence to: Silvia Vidal, Department of Immunology, Institut Rec. Hospital Sant Pau, Avda. Antoni M. Claret, 167. Barcelona 08025, Spain. svidal@santpau.es
Telephone: +34-93-2919017 Fax: +34 -93-2919066
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Abstract

AIM: To examine genetic variation of nucleotide oligomerization domain 1 (*NOD1*) and *NOD2*, their respective influences on Crohn's disease phenotype and gene-gene interactions.

METHODS: (*ND1+32656*1*) *NOD1* polymorphism and *SNP8*, *SNP12* and *SNP13* of *NOD2* were analyzed in 97 patients and 50 controls. *NOD2* variants were determined by reaction restriction fragment length polymorphism analysis. *NOD1* genotyping and *NOD2* variant confirmation were performed by specific amplification and sequencing.

RESULTS: The distribution of *NOD1* polymorphism in patients was different from controls (P = 0.045) and not altered by existence of *NOD2* mutations. In this cohort, 30.92% patients and 6% controls carried at least one *NOD2* variant (P < 0.001) with R702W being the most frequent variant. Presence of at least one *NOD2* mutation was inversely associated with colon involvement (9.09% with colon *vs* 36.4% with ileal or ileocolonic involvement, P = 0.04) and indicative of risk of penetrating disease (52.63% with penetrating *vs* 25.64% with non-penetrating or stricturing behavior, P = 0.02). L1007finsC and double *NOD2* mutation conferred the highest risk for severity of disease (26.3% with penetrating disease *vs* 3.8% with non-penetrating or stricturing behavior presented L1007finsC, P = 0.01 and 21.0% with penetrating disease *vs* 2.5% with non-penentrating or stricturing behavior carried double *NOD2* mutation, P = 0.007). Exclusion of patients with *NOD2* mutations from phenotype/*NOD1*-genotype analysis revealed higher prevalence of *1*1 genotype in groups of younger age at onset and colonic location.

CONCLUSION: This study suggests population differences in the inheritance of risk *NOD1* polymorphism and *NOD2* mutations. Although no interaction between *NOD1*-*NOD2* was noticed, a relationship between disease location and Nod-like receptor molecules was established.

Key words: Crohn's disease; Nucleotide oligomerization domain 1; Nucleotide oligomerization domain 2

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. Although the etiopathogenesis of this disease remains poorly understood, both genetic and environmental factors have been suggested to predispose to CD. Various disease phenotypes, including age at diagnosis, sex, family history, location of disease, response to medical therapies and behavior of the disease may be genetically determined.

Experimental and observational data suggest that intestinal inflammation arises from abnormal immune reactivity to bacterial flora in the intestine of individuals who are genetically predisposed[1]. The analysis of the molecules that participate in the response of commensal organisms revealed that gastric and intestinal cells are

largely deficient in TLR signaling and must rely on alternative systems, such as Nod-like receptors (NLRs) for the detection of pathogens. The mammalian NLR family is composed of more than 20 members that share a modular domain organization of a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding site domain and a N-terminal protein-protein-interaction domain composed of a CARD (caspase activation and recruitment domain), pyrin domain or Bir domain[2].

The first NLRs reported to have a direct function as intracellular pattern recognition molecules were Nucleotide oligomerization domain 1 (*NOD1*) (*CARD4*) and *NOD2* (*CARD15*); both proteins detect distinct substructures from bacterial peptidoglycan. *NOD1* detects a unique tripeptide motif found in Gram-negative bacterial peptidoglycan and also in specific Gram-positive bacteria such as *Listeria* and *Bacillus* spp[3]. *NOD2* detects muramyl dipeptide, the largest molecular motif common to Gram-negative and Gram-positive bacteria[4]. It is expressed in intestinal epithelial cells, with high expression in Paneth cells in the small intestine, intestinal myofibroblasts, granulocytes, endothelial, and monocyte-derived cells[5,6].

Identification of *NOD2* as the first susceptibility gene for CD was a breakthrough in understanding inflammatory bowel disease (IBD) pathogenesis. *NOD2* gene is located at the CD susceptibility locus (*IBD1*) on chromosome 16q12[7,8] and it has more than 60 sequence variants. Although, disease-associated *NOD2* mutations linked to Blau syndrome and early onset of sarcoidosis have been found in the region encoding the nucleotide-binding site domain[9,10], the three common genetic mutations linked to CD are mapped within or adjacent to the LRR region of *NOD2* (leading to protein changes at R702W, G908R, L1007finsC)[7,8]. These mutations are associated with an altered NF-κB activation and the linkage is particularly strong with ileal and ileocolonic CD[11,12]. *NOD2* variants are associated with early surgery due to stenosis, postsurgical recurrence, familial CD[13] and stricturing and penetrating forms of CD[14].

CD association with *NOD2* has been widely replicated. However, investigations into the inheritance of the three risk alleles in *NOD2* associated with susceptibility to CD have demonstrated a remarkable heterogeneity across ethnicities and populations with regional variation across Europe[15,16].

The discovery of *NOD2*-related innate immune defects in certain CD cases has led to speculation about defects in other pattern recognition receptors and downstream signaling molecules. The gene encoding *NOD1* (*CARD4*) is located within the chromosome 7p14 IBD locus, a region that contains an IBD susceptibility locus in British families[17]. An association between a complex insertion/deletion polymorphism (*ND1+32656*1*) in *NOD1* and susceptibility to IBD has been described. Particularly, this polymorphism has been associated to age at diagnosis and to the presence of IBD extraintestinal manifestations[18]. This *NOD1* polymorphism has also been associated to increased susceptibility to asthma[19,20]. In both diseases, the mutation has been found to be an insertion/deletion polymorphism in an intron of *NOD1*. Convincing replication of these findings is pending, since no evidence of association between *ND1+32656*1* and IBD was found in two recent well-powered data sets[21,22].

The present study examines the genetic variation in *NOD1* and *NOD2* and their respective influences on the CD phenotype (age at diagnosis, disease location and behavior) in a cohort of well-characterized CD patients. Since *NOD1* and *NOD2* share structure and functions, a potential interaction between *NOD1* and *NOD2* variants in CD phenotype was analyzed. After stratifying patients by their *NOD2* genotype, the distribution of *NOD1* polymorphism was determined and the contribution of each genotype was studied in regard to the disease phenotype.

MATERIALS AND METHODS

Patients

Ninety-seven CD patients attending the IBD outpatient clinic of Hospital Sant Pau (Barcelona, Spain) were prospectively included in the study. Fifty healthy controls matched for age, sex and geography were also evaluated. CD diagnoses were based on clinical, radiologic, endoscopic and pathologic bases. Patients with CD were classified according to Montreal classification for age at onset, disease location and behavior[23]. All patients and healthy controls gave informed consent and the study was approved by the local ethics committee.

Genotyping

Analysis of *NOD2* variants was performed as previously described, using genomic DNA extracted from blood samples by Qiagen kit (Qiagen, Heiden, Germany). A panel of 3 single nucleotide polymorphisms (*SNP8*, *12* and *13*) was detected by a polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis (PCR-RFLP)[7]. Each *NOD2* variant was initially amplified by PCR using specific primers (Table 1). The PCR products were subsequently analyzed by restriction enzyme cleavage and gel electrophoresis. For assay of the SNP8, the PCR product (185 bp) was digested with MspI, resulting in the following fragments: 20, 35, 54 and 76 bp in R702 homozygous; 20, 35 and 130 bp in 702W homozygous and 20, 35, 54, 76, and 130 bp in heterozygous. For assay of the SNP12, the PCR product (163 bp) was digested with HhaI, resulting in the following fragments: 163 bp in G908 homozygous; 27 and 136 bp in 908R homozygous and 27, 136 and 163 in heterozygous. In order to detect the SNP13, the PCR product (151 bp) was digested with ApaI, resulting in the following fragments: 151 bp for Leu1007 homozygous; 20 and 131 bp in 1007Pro homozygous and 20, 131 and 151 bp in heterozygous.

Genotyping of *NOD1* (*ND1+32656*) polymorphism and confirmation of the three *NOD2* mutations were performed by specific amplification with the primers described in Table 1 and the subsequent sequencing of the amplified products. Sequencing reaction was performed using ABI PRISM BigDye terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed by Genescan analysis on an ABI Prism 3100 Genetic Analyser according to the manufacturer's protocol (Applied Biosystem).

Table 1 Primers for *NOD2* and *NOD1* genotyping

	Forward	Reverse	Size (bp)
NOD2			
R702W	5′-AGATCACAGCAGCCTTCCTG-3′	5′-CACGCTCTTGGCCTCACC-3′	185
G908R	5′-CTCTTTTGGCCTTTTCAGATTCTG-3′	5′-CAGCTCCTCCCTCTTCACCT-3′	163
L1007finsC	5′-GGCAGAAGCCCTCCTGCAGGGCC-3′	5′-CCTCAAAATTCTGCCATTCC-3′	151
NOD1			
ND1+32656	5′-TGACTGTGTGTGACTCTCTCTGC-3′	5′-TGGTGAAAGCTCTCCACTATCTC-3′	250

Table 2 Genotype at *NOD2* polymorphisms in CD cases and healthy controls

Mutation	Group	Genotype count, *n* (%)			OR (95% CI)[1]	*P*[2]
		WT/WT	Heterozygous	Homozygous		
R702W	CD	75 (77.32)	21 (21.65)	1 (1.03)	7.04 (1.58-31.30)	0.004
	Controls	48 (96.00)	2 (4.00)	0		
G908R	CD	92 (94.85)	5 (5.15)	0		0.166
	Controls	50 (100)	0	0		
L1007finsC	CD	89 (91.75)	8 (8.25)	0	4.40 (0.53-36.25)	0.167
	Controls	49 (98.00)	1 (2.00)	0		

[1]ORs and probability values for disease status associated with carriage of at least 1 mutant allele (heterozygous, compound heterozygous and homozygous were grouped together). [2]*P*-values were calculated with the Fisher's Exact test when comparing controls and CD patients.

Statistical analysis

Genotype and allele frequencies of the patients and controls were compared by the χ^2 test or Fisher exact test in 2×2 contingency tables with at least 1 expected value < 5. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to estimate relative risks. A two-tailed *P* value $\leqslant 0.05$ was considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 14.0 for Windows (SPSS Inc., Chicago, Ⅲ).

RESULTS

Frequencies of three NOD2 mutant alleles and one NOD1 polymorphism in CD patients and healthy controls

NOD2 gene mutations (R702W, G908R and L1007finsC) were determined in 97 CD patients and 50 healthy controls. Frequencies are summarized in Table 2. The distribution of genotypes at each mutation was significantly different in CD patients versus controls. R702W was the most frequent variant in CD and controls (21.65% and 4%, respectively, $P = 0.004$), and the only homozygous mutant patient in this cohort was found for this SNP8. Carriage of R702W was associated to the highest risk for CD in our cohort of patients (OR = 7.04; 95% CI: 1.58-31.30). Genotype frequency of the L1007finsC variant was lower than R702W, but showed a tendency to be higher in CD patients than in controls (8.25% *vs* 2%, $P = 0.167$; OD 4.40, 95% CI: 0.53-36.25). The *NOD2* variant with the lowest frequency in CD patients and in controls was G908R (5.15% *vs* 0%, $P = 0.166$). No homozygous *NOD2* mutant was found for L1007finsC or G908R. In this CD cohort, 30.92% of patients carried at least one variant of *NOD2* compared with 6% of healthy controls ($P < 0.001$) (Table 3), conferring a high risk for CD (OR 7.01; 95% CI: 2.02-24.30). Six CD patients but no controls carried two *NOD2* variant alleles.

NOD1 complex insertion/deletion polymorphism (*ND1+32656*) was examined in the same cohort of patients and controls (Table 4). Fifty-two percent of controls were *1*1, 34% were *1*2 and 14% were *2*2, whereas 59.79% of CD patients were *1*1, 37.11% were *1*2 and only 3.09% were *2*2. The distribution of *NOD1* genotype according to the *ND1+32656* polymorphism in CD patients and controls was statistically different ($P = 0.045$). Frequency of CD patients carrying *1 allele was 96.8% whereas in controls it was 86%, conferring a significant risk to develop the disease (OR 5.10; 95% CI: 1.25-20.68, $P = 0.032$).

Distribution of *NOD1* genotype according to WT or mutant *NOD2* was analyzed in CD patients to assess potential interactions between NOD1 and NOD2 (Table 4). Among those patients carrying at least one *NOD2* mutant allele, 60% of the patients were *1 *1, 36.66% were *1 *2 and 3.33% were *2 *2. Similarly, 59.70% of NOD2 WT/WT were *1 *1, 37.31% were *1 *2 and 2.98% were *2 *2. The presence of *NOD2* mutant alleles had therefore no influence on the *NOD1* polymorphism distribution ($P = 0.99$), suggesting no gene-gene interactions.

Clinical characteristics of CD patients according to the NOD2 genotype

CD patients were classified according to Montreal classification, with minor modifications as indicated in Table 5. The association of *NOD2* mutations to each CD phenotype was analyzed using each mutant genotype. The presence of at least one risk allele or the joint analysis of

Table 3 Distribution of *NOD2* mutations in CD patients and controls

NOD2 genotype	CD Patients, *n* (%)	Controls, *n* (%)	OR (95% CI)[1]	*P*[2]
At least one variant[3]	30 (30.92)	3 (6)	7.01 (2.02-24.30)	< 0.001
Heterozygous	24 (24.74)	3 (6)	5.61 (1.59-19.0)	0.003
Compound heterozygous[4]	5 (5.15)	0		
Homozygous	1 (1.03)	0		

[1]ORs and probability values for disease status associated with *NOD2* genotype. [2]*P*-values are calculated with the Fisher's Exact test when comparing controls and CD patients. [3]At least one variant was considered if any subject had at least one copy of the variant allele. [4]Compound heterozygous was defined as the presence of two different variants.

Table 4 Genotype frequencies of *ND1+32656* in CD patients and controls

Group	*NOD2*	*n*[1]	*NOD1* genotype, *n* (%)		
			*1*1	*1*2	*2*2
CD		97	58 (59.79)[2]	36 (37.11)	3 (3.09)
	WT/WT	67	40 (59.70)	25 (37.31)	2 (2.98)
	Mutant[3]	30	18 (60.00)	11 (36.66)	1 (3.33)
Controls		50	26 (52.00)	17 (34.00)	7 (14.00)
	WT/WT	47	23 (48.93)	17 (36.17)	7 (14.89)
	Mutant	3	3 (100)	0	0

CD: Crohn's disease; WT: Wildtype. [1]Number of CD patients or controls; [2]Results are expressed as number of CD patients (% of CD patients); [3]NOD2 mutant was considered any subject that inherited at least one copy of the variant allele.

compound heterozygous and homozygous for *NOD2* mutations were considered as a single independent variable.

A high proportion of patients in this cohort were diagnosed under the age of 40 (A1 + A2, *n* = 2 + 78), whereas 17 patients were diagnosed over 40 years (A3). All 6 patients with 2 mutant *NOD2* alleles were diagnosed before 40 years of age.

To study the association between genotype and disease location, one L1 + L4 patient was included in the L1 group (*n* = 34, 35.05%), and another L2 + L4 patient was included in the L2 group (*n* = 23, 23.71%). *NOD2* WT/WT patients were similarly distributed in L1, L2 and L3 groups: in 21.65% patients disease location was terminal ileum (L1), in 20.62%, it was colonic (L2), and in 26.80%, it was ileocolonic (L3). However, location of disease in *NOD2* mutant patients was not identical to *NOD2* WT/WT patients (*P* = 0.08). Despite the fact that R702W polymorphism was the most frequent in this cohort, only one patient with colon location carried this mutation (the patient had a double *NOD2* mutation). The presence of at least one mutant *NOD2* gene was inversely associated with exclusively colonic involvement (L2) (*P* = 0.04, OR 0.26; 95% CI: 0.07-0.96). When analyzing compound heterozygous and homozygous *NOD2* mutations, 2.06% of patients were L1, 1.03% of patients were L2 and 3.09% of patients were L3, indicating a comparable distribution.

Similarly to location, behavior groups in CD patients were simplified as follows: B1 group included 4 patients B1p (*n* = 37, 38%), B2 included 4 patients B2p (*n* = 41, 42%) and B3 included 4 patients B3p (*n* = 19, 19.5%). Distribution of *NOD2* mutations was different depending on disease behavior (*P* = 0.003). The presence of at least one mutant *NOD2* allele was indicative of risk of penetrating disease (B3) (*P* = 0.02, OR 3.22, 95% CI: 1.14-9.06) with the allele L1007finsC being indicative of the highest risk (*P* = 0.007, OR 8.92; 95% CI: 1.91-41.68). The frequency of double *NOD2* mutants was significantly higher in the B3 group than in the B2 and B1 groups (66% of the double *NOD2* mutants were B3, 33.3% were B2 and non-double *NOD2* mutants were B1). Presence of two *NOD2* mutant alleles was therefore indicative of risk for severity of disease (*P* = 0.01, OR 10.13; 95% CI: 1.70-60.40). The exam of the behavior through the course of disease showed an expected changing pattern[24]. There was a progressive reduction in the proportion of patients in the group B1 (51.6% patients with 5 years of disease, 34.9% patients with 6-9 years of disease and 21.7% patients with 10-15 years of disease). Inversely, there was a progressive increase in the proportion of patients in the groups of more complicated forms, B2 (34.5% patients with 5 years of disease, 44.2 patients with 6-9 years of disease and 52.2% patients with 10-15 years of disease) and B3 (13.8% patients with 5 years of disease, 20.9% patients with 6-9 years of disease and 26.1% patients with 10-15 years of disease). Selecting the group of patients with 6-9 years of disease (*n* = 43), the presence of at least one NOD2 mutation was indicative of risk of penetrating disease (the B3 group) (*P* = 0.046, OR = 5.55, 95% CI: 1.14-27.01) and 66.7% of the double *NOD2* mutants were included in the B3 group. Twelve patients with perianal disease (B1p, B2p, and B3p) were analyzed separately. Four of these patients presented one *NOD2* mutation, one was a compound heterozygous and the rest were *NOD2* WT/WT.

Clinical characteristics of CD patients according to the NOD1 genotype

Phenotype of CD patients was analyzed according to the *ND1+32656* polymorphism of *NOD1* gene. Distribution of *NOD1* genotype according to older age at diagnosis (A3 *P* = 0.64), location (L1 *P* = 0.28, L2 *P* = 0.56 and L3 *P* = 0.26) and behavior (B1 *P* = 0.55, B2 *P* = 0.99 and B3 *P* = 0.99) of the disease were not different from healthy controls. Similarly to a previous report, ND1+32656 genotype distribution in the group of early-onset CD (A1 + A2) was different from that observed in healthy controls. Only 2.5% of CD patients in the early-onset group had *2*2 genotype compared to 14% of healthy controls (*P* = 0.04).

Since *NOD2* mutations have a strong association with some CD clinical characteristics, and in particular with the ileal location, 30 CD patients that presented at least one *NOD2* mutation were excluded from the phenotype/genotype study to prevent any influence of *NOD2* (Table 6). When comparing the distribution of *NOD1*

Table 5 Clinical characteristics of CD patients according to *NOD2* genotype

Clinical features	*n*[1]	*NOD2* genotype, *n* (%)					*P*[2] OR (95% CI)
		WT/WT	R702W/WT	G908R/WT	L1007fsinsC/WT	Heter.compound & homozygous	
Age at diagnosis							
< 40 yr (A1 + A2)	80	54 (55.67)[3]	13 (13.40)	3 (3.09)	4 (4.12)	6 (6.18)	0.57 1.56 (0.46-5.27)
> 40 yr (A3)	17	13 (13.40)	3 (3.09)	0	1 (1.03)	0	
Location							
Ileal (L1)	34	21 (21.65)	8 (8.25)	1 (1.03)	2 (2.06)	2 (2.06)	0.26 1.67 (0.69-4.06)
Colonic (L2)	23	20 (20.62)	0	1 (1.03)	1 (1.03)	1 (1.03)	0.04 0.26 (0.07-0.96)
Ileocolonic (L3)	40	26 (26.80)	8 (8.25)	1 (1.03)	2 (2.06)	3 (3.09)	0.5 1.38 (0.57-3.29)
Behavior							
Non-stricturing, non-penetrating (B1)	37	24 (24.74)	8 (8.25)	3 (3.09)	2 (2.06)	0	0.5 1.37 (0.56-3.29)
Stricturing (B2)	41	34 (35.05)	5 (5.15)	0	0	2 (2.06)	0.01 0.29 (0.11-0.78)
Penetrating (B3)	19	9 (9.28)	3 (3.09)	0	3 (3.09)	4 (4.12)	0.02 3.22 (1.14-9.06)

CD: Crohn's disease; WT: Wildtype. [1]Number of CD patients in each subgroup; [2]*P*-values, odds ratios and confidence intervals refer to the comparison of presence versus absence of the at least one mutant *NOD2* allele; [3]Results are expressed as number of CD patients (% of CD patients).

polymorphism in each phenotype group with healthy controls, a higher prevalence of *1*1 was observed in the group A1 + A2 ($P = 0.04$). Interestingly, there was a clear tendency of the colonic group (L2) to have a higher frequency of *1*1 and lower frequency of *1*2 and *2*2 than controls, but the distribution of the *ND1+32656* polymorphism was not statistically different because the low number of cases decreased the power of the test. Distribution of this *NOD1* polymorphism in the other clinical subgroups of CD patients was comparable to healthy controls. Seven of the 12 patients with perianal disease (B1p + B2p + B3p) were *NOD2* WT/WT. In this subgroup of patients, *NOD1* polymorphism analysis showed that three of them were *1*1 and four were *1*2.

Table 6 Clinical characteristics of *NOD2* WT/WT CD patients according to *NOD1* genotype

Clinical features	*n*[1]	*NOD2* WT/WT		
		NOD1: *1*1	*1*2	*2*2
Age at diagnosis				
< 40 yr (A1 + A2)	54	62.96[2]	35.18	1.85
> 40 yr (A3)	13	46.15	46.15	7.69
Location				
Ileal (L1)	21	57.14	38.09	4.76
Colonic (L2)	20	70	25	5
Ileocolonic (L3)	26	53.84	46.15	0
Behavior				
Non-stricturing, non-penetrating (B1)	24	62.5	33.33	4.16
Stricturing (B2)	34	61.76	35.29	2.94
Penetrating (B3)	9	44.44	55.55	0
Controls	47	48.93	36.17	14.89

WT: Wildtype. [1]Number of CD patients or controls in each subgroup; [2]Values are expressed as the percent of patients in each clinical subgroup.

DISCUSSION

The frequency of *NOD2* mutant alleles associated to CD in our cohort of patients was within the European range, but deviated somewhat from populations of nearby geographic regions[25,26]. The frequency of R702W was one of the highest described in Caucasian populations, whereas the frequency of L1007finsC was lower than in other studies[27]. This observation is consistent with marked racial and regional differences described in the inheritance of the three risk *NOD2* alleles[16].

As expected, carriage of *NOD2* mutations conferred a high risk for developing CD, but this was neither necessary nor sufficient for CD development. The three *NOD2* mutations were not equally involved in CD susceptibility. The presence of R702W showed the strongest risk for CD in our cohort of patients. However, this mutation was not associated to CD in Galician, Finnish or Scottish populations[26]. The mutation with the strongest CD association in several familial and non-familial studies was L1007finsC[14], but this was not so in our cohort. Although the frequency of L1007finsC was noticeably elevated in CD patients, the absence of controls with this genotype precluded a statistical comparison.

We found an association between the polymorphism located at the intron IX-exon IX boundary of *NOD1* and susceptibility to CD in our cohort of patients. These results confirm a previous report associating this *NOD1* polymorphism with early IBD-onset and extraintestinal manifestations[18]. Although one recent study did not show a significant association with IBD[21], this *NOD1* non-coding polymorphism showed a strong association with asthma and the presence of elevated IgE levels in three independent panels of subjects[20]. Other *NOD1* polymorphisms in the coding sequence have been previously examined and showed no influence in CD susceptibility[28]. Mutations with phenotypic effects should be predominantly found at the coding sequence but complex disease susceptibility is often mediated through regulatory polymorphisms. In this case, *ND1+32656* may affect the binding of an unknown nuclear factor[20]. The involvement of *NOD1* gene is not surprising, since *NOD1*, similarly to *NOD2*, is involved in the recognition of intracellular bacterial pathogen-associated molecular patterns[29] and the two molecules share structure and functional similarities. Certain polymorphisms and

mutations in these molecules may, therefore, result in abnormalities during bacterial recognition with direct implications for CD pathogenesis. Given the importance of these results, further confirmatory studies are warranted in more and larger IBD populations. In order to maximize the opportunities to compare clinical subgroups, location was kept simple and genotyping was specifically blinded to clinical status. Mutations of the *NOD2* gene were rare among our patients with disease limited to the colon (L2). This is in accordance with recent studies showing that *NOD2* mutations (particularly L1007finsC) are strongly related to an increased risk of developing ileal CD. In our cohort of patients we only found this association after combining ileal and ileocolonic patients. This could be the consequence of the low rates of limited ileal CD in our cohort of patients compared to other studies (ranging from 40% to 50% in CD patients)[25,26]. Since location remains relatively stable during the course of the disease[24], the low rates of ileal CD seen in our patients could be attributable to the impact of interobserver disagreement[30], variation of disease location among different backgrounds[31] and even differences in diagnostic techniques. The present study suggests a relationship between disease location and different Nod-like receptor molecules, with relevant clinical implications. Distinctive subcellular location, trafficking, and expression of each Nod-like receptors could be confining the association of *NOD1* and *NOD2* with location of the disease at different parts of the gastrointestinal tract. In healthy humans, *NOD2* is expressed in Paneth cells within the crypts of the small intestine but not in colonic epithelium[6]. On the other hand, colon intestinal epithelial cells constitutively express *NOD1*[32]. *NOD1* or *NOD2* prevalence in colon or ileum could also be due to the predominance of different intracellular organisms or enteroinvasive bacteria for which they are receptors[33]. Further studies are needed to better clarify this subject.

A higher genetic load of *NOD2* mutations increased the susceptibility to CD and determined an aggressive course of the disease. Although CD behavior is a dynamic process progressing towards complicated forms in 80% of patients[24], the presence of *NOD2* variants could predict a stricturing and penetrating disease[14]. In addition, *NOD2* variants have been associated with early surgery due to stenosis and with CD recurrence after surgery[13]. No association was established between the *NOD1* polymorphism and disease behavior.

When comparing these results with other published genotype/phenotype associations, potential confounding factors should be taken into account to understand the differences. Agreement in Montreal classification, modification of the phenotype during follow-up, as well as the mixture of populations in some studies could be masking the particularities of each population. Our study adds two novel approaches to previous studies. First, two functionally related genes were analyzed for the first time in the same population, and second, the association phenotype/*NOD1* genotype was established after ruling out the strong influence of *NOD2*. Although this work emphasized the importance of *NOD1* and *NOD2* on CD disease phenotype, the complexity of IBD genetics should not be ignored. Individual combinations of genetic risk factors from other molecules such as OCTN, DLG5, TUCAN, MDR1, TNF and TLRs[34-40] would depict a specific clinical picture for each CD patient.

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COMMENTS

Background

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. Genetic and environmental factors have been suggested to predispose to CD. CD association with *NOD2* mutations has been widely replicated but with remarkable heterogeneity across populations. Similarly to *NOD2*, *NOD1* has a direct function as intracellular pattern recognition molecules but detecting different substructures from bacterial peptidoglycan. The present study examines the genetic variation in *NOD1* and *NOD2* and their respective influences on the CD phenotype in a cohort of well-characterized CD patients.

Research frontiers

Individual combinations of genetic risk factors from *NOD2*, *NOD1* and other molecules, such as OCTN, TNF and TLRs, would depict a specific clinical picture for each CD patient.

Innovations and breakthroughs

This study adds two novel approaches to previous studies. First, *NOD2* and *NOD1* were analyzed for the first time in the same population and second, the association phenotype/*NOD1* genotype was established after ruling out the strong influence of *NOD2*. The present results suggest a relationship between disease location and different Nod-like receptor molecules.

Applications

This is an association study that compares the allele or genotype frequencies of two genes between affected and unaffected individuals of Crohn's disease. Exploring new gene variants associated with inflammatory bowel disease would make possible the identification of proteins located in certain pathophysiological pathways.

Terminology

NODs are cytosolic proteins that contain a nucleotide-binding oligomerization domain (NOD). As sensors of bacterial components, *NOD1* and *NOD2* are triggered by host recognition of specific motifs in bacterial peptidoglycan and, upon activation, induce the production of proinflammatory mediators.

Peer review

This is a very well written paper. Authors examined genetic variation of *NOD1* and *NOD2*, their respective influences on Crohn's disease phenotype and gene-gene interactions. This study suggests population differences in the inheritance of risk *NOD1* polymorphism and *NOD2* mutations.

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RAPID COMMUNICATION

An optimized ^{13}C-urea breath test for the diagnosis of *H pylori* infection

Germán Campuzano-Maya

Germán Campuzano-Maya, Professor, Ad honorem, School of Medicine, University of Antioquia, Medellín, Colombia; Medical Director, Laboratorio Clínico Hematológico®, Medellín, Colombia
Correspondence to: Dr. Germán Campuzano-Maya, Laboratorio Clínico Hematológico, Carrera 43C No. 5-33, Medellín, Colombia. gcampuzano@hematologico.com
Telephone: +574-4444200 Fax: +574-3128232
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Abstract

AIM: To validate an optimized ^{13}C-urea breath test (^{13}C-UBT) protocol for the diagnosis of *H pylori* infection that is cost-efficient and maintains excellent diagnostic accuracy.

METHODS: 70 healthy volunteers were tested with two simplified ^{13}C-UBT protocols, with test meal (Protocol 2) and without test meal (Protocol 1). Breath samples were collected at 10, 20 and 30 min after ingestion of 50 mg ^{13}C-urea dissolved in 10 mL of water, taken as a single swallow, followed by 200 mL of water (pH 6.0) and a circular motion around the waistline to homogenize the urea solution. Performance of both protocols was analyzed at various cut-off values. Results were validated against the European protocol.

RESULTS: According to the reference protocol, 65.7% individuals were positive for *H pylori* infection and 34.3% were negative. There were no significant differences in the ability of both protocols to correctly identify positive and negative *H pylori* individuals. However, only Protocol 1 with no test meal achieved accuracy, sensitivity, specificity, positive and negative predictive values of 100%. The highest values achieved by Protocol 2 were 98.57%, 97.83%, 100%, 100% and 100%, respectively.

CONCLUSION: A 10 min, 50 mg ^{13}C-UBT with no test meal using a cut-off value of 2-2.5 is a highly accurate test for the diagnosis of *H pylori* infection at a reduced cost.

Key words: *H pylori*; ^{13}C-urea breath test; Diagnosis; Accuracy; Cost

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INTRODUCTION

H pylori infection is present in around 50% of the world population[1], with higher prevalence rates in developing countries where it is the most frequent chronic infection in human kind[2].

H pylori infection has been associated with the pathogenesis of gastric disorders such as gastritis, duodenal and gastric ulcer, gastric cancer and MALT lymphoma[3], and a variety of extradigestive disorders including hematologic, such as iron deficiency anemia[4], pernicious anemia[5], autoimmune neutropenia[6], Schönlein-Henoch purpura[7], thrombotic thrombocytopenic purpura[8] and idiopathic thrombocytopenic purpura[9]. It has also been implicated in the pathogenesis of traditional autoimmune diseases, including rheumatoid arthritis[10], Sjögren syndrome[11] and autoimmune thyroiditis[12], dermatologic diseases such as rosacea[13] and urticaria[14], and cardiovascular events[15,16] among others.

Diagnosis of *H pylori* infection can be established by either invasive or non-invasive techniques. Invasive techniques, by means of endoscopy, are expensive[17], cause patient discomfort and introduce the risk of cross-infection[18,19]; moreover, there is morbidity and mortality associated with the procedure[20] and is not indicated in all cases where the *H pylori* status must be determined[21,22]. Non-invasive methods include serology[23] ^{14}C-urea or ^{13}C-urea breath test (UBT)[24,25], stool antigen test[26] and blood urea test[27].

The principle of the ^{13}C-UBT relies upon the ability of the urease, produced by *H pylori* in the gastric mucosa, to hydrolyze the orally administered ^{13}C-urea. This enzyme breaks down any urea in the stomach to ammonia and carbon dioxide (CO_2), which is absorbed into the blood stream and then released from the lungs. The labelled carbon dioxide ($^{13}CO_2$) is detected in breath samples[28].

The aim of the present study was to standardize and validate an assay that is cost-effective, while preserving excellent diagnostic accuracy. Two simple protocols were validated against the standard European protocol[29], which included modifications in the dose, formulation and *via* of urea administration, sample collection times and test meal. Appropriate cut-off values for these assays were also established.

MATERIALS AND METHODS

Subjects

The study population included 70 volunteers with no gastrointestinal symptoms. The volunteers were informed about the study and the tests, and signed an informed consent in accordance with the Helsinki Declaration[30]. The study was classified as a research study with no biological, physiological, psychological or social risks by the Health Ministry of Colombia[31]. Because of the nature of the study in healthy volunteers, it was considered non-ethical to perform invasive tests such as biopsy, culture or endoscopy.

Protocols

Reference protocol: *H pylori* infection status of individuals was determined by the ^{13}C-UBT, according to the European protocol described before[29] and using commercial kits (TAU-KIT, Isomed SL, Madrid, Spain) that provide both a sensitivity and specificity close to 100%[25]. This protocol was standardized and was validated for our region, with over 15000 assays performed, and used as the gold standard. The ^{13}C-UBT was analyzed by means of continuous flow-isotope ratio mass spectrometry (ABCA, SerCon, Cheshire, UK) at the Laboratorio Clínico Hematológico® in Medellín, Colombia.

The reference protocol was performed as follows: After fasting for at least 8 h, individuals were given 4.2 g of citric acid dissolved in 200 mL of water. Ten minutes later, a duplicate basal breath sample was collected. Immediately after, individuals were given 100 mg of ^{13}C-urea dissolved in 125 mL of water. After 30 min, a duplicate post-urea breath sample was collected. Results over 2.5 delta-over-baseline (DOB) were considered positive for *H pylori* infection.

Protocol 1: After fasting for at least 8 h, a first basal breath sample was collected. Individuals were given 50 mg of ^{13}C-urea (99%, Isotec, Miamisburg, Ohio, USA) dissolved in 10 mL of water, taken as a single swallow. Immediately after, individuals were given 200 mL of water (pH 6.0). Volunteers, with a final volume of 210 mL, were asked to make a circular motion around the waistline for a few times to homogenize the aqueous solution and allow contact of the ^{13}C-urea with the entire gastric mucosa. Additional breath samples were collected afterwards at 10, 20 and 30 min.

Protocol 2: Same as Protocol 1, except that 4.2 g of dehydrated citric acid were added to the 200 mL of water.

The performance of both protocols was analyzed at various cut-off values from 0.5 to 5.5, at the different time intervals (10, 20 and 30 min).

Statistical analysis

The χ^2 test was used to analyze associations between qualitative variables. For quantitative variables, the Wilcoxon's signed rank sum tests and Student's *t*-test were applied. Normality of the distribution of the data was assessed with the Wilk-Shapiro test. Sensitivity, specificity, positive predictive value, negative predictive value, accuracy, Youden index, likelihood ratios for a positive (LR+ve) or negative (LR-ve) test were calculated against the defined gold standard. The effectiveness of each protocol was evaluated by ROC analysis. Processing and analysis of data were done with the SPSS (Statistical Product for Service Solutions) version 12.0 and EPIDATE Version 3.0. A value of $P < 0.05$ was considered statistically significant.

RESULTS

This study included 70 individuals, 24 (34.3%) males and 46 (65.7%) females, with an average age of 39.63 (SD ± 12.58) years for males and 34.33 (SD ± 10.17) years for females. There were no significant differences between the mean age for males and females ($P = 0.061$). According to the reference protocol, 46 (65.7%) individuals were positive for *H pylori* infection and 24 (34.3%) were negative. When assessed by gender, 17 (70.8%) males and 29 (63%) females were positive for *H pylori*; this association was not statistically significant ($P = 0.515$).

Table 1 shows the performance of the protocols in terms of sensitivity, specificity, accuracy, positive and negative predictive values, Youden index and likelihood ratios for a positive (LR+ve) or (LR-ve) test with the different DOB cut-off values at 10, 20 and 30 min. Only Protocol 1 (with no test meal) achieved accuracy, sensitivity, specificity, positive and negative predictive values of 100%. The highest values achieved by Protocol 2 were 98.57%, 97.83%, 100%, 100% and 100%, respectively.

There were no significant differences in the ability of both protocols to correctly identify positive and negative *H pylori* individuals at 10 ($P = 0.32$), 20 ($P = 0.32$) and 30 min ($P = 0.32$). These results were confirmed by ROC analysis (Figure 1). The areas under the ROC curves for both protocols were as follows: for Protocol 1, 1.0 at 10, 20 and 30 min; for Protocol 2, 0.9837 at 10 and 30 min, and 0.9873 at 20 min. Although these results were not statistically different, Protocol 1 shows the maximum optimal values for an assay.

Table 2 shows the distribution of the DOB values at 10, 20, 30 min for *H pylori* positive and negative individuals for Protocols 1 and 2. For Protocol 1, the median DOB for *H pylori* infected individuals at 10 min was 13.64, while for Protocol 2 was 12.02. There was no statistically significant difference between these 2 values (Wilcoxon, $P = 0.121$). In contrast, median DOB values at 20 and 30 min for both protocols showed significant differences ($P = 0.006$ and $P = 0.001$, respectively). In addition, for non-infected individuals there were no statistically significant differences in the median DOB values at 10, 20 and 30 min ($P = 0.710$, $P = 0.440$ and $P = 0.346$, respectively) between both protocols.

DISCUSSION

The ^{13}C-UBT has become the gold standard of the non-invasive tests for diagnosing *H pylori* infection, before and after eradication treatment. Recently, The Maastricht Ⅲ Consensus Report has recommended the ^{13}C-UBT as the best option to establish the diagnosis of *H pylori* infection, especially in patients in whom endoscopy is not indicated[22].

Table 1 Performance of protocols (P1 and P2) in terms of sensitivity, specificity, accuracy, positive and negative predictive values, Youden index and likelihood ratios for a positive (LR+ve) or (LR-ve) test with the different DOB cut-off values at 10, 20 and 30 min

Time (min)	DOB	Sensitivity		Specificity		Accuracy		Positive predictive value		Negative predictive value		Youden index		LR+ve		LR-ve	
		P1	P2	P1	P2	P1	P2	P1	P2	P1	P2	P1	P2	P1	P2	P1	P2
10	0.5	100	97.83	45.83	62.5	81.43	85.71	77.97	83.33	100	93.75	0.46	0.6	1.85	2.61	[1]	0.03
	1.0	100	97.83	83.33	79.17	94.29	91.43	92	90.0	100	95	0.83	0.77	6.00	4.7	[1]	0.03
	1.5	100	97.83	95.83	95.83	98.57	97.14	97.87	97.83	100	95.83	0.96	0.94	24.00	23.48	[1]	0.02
	2.0	100	97.83	100	100	100	98.57	100	100	100	96	1.00	0.98	[2]	[2]	[1]	0.02
	2.5	100	95.65	100	100	100	97.14	100	100	100	92.31	1.00	0.96	[2]	[2]	[1]	0.04
	3.0	97.83	95.65	100	100	98.57	97.14	100	100	96.0	92.31	0.98	0.96	[2]	[2]	0.02	0.04
	3.5	97.83	95.65	100	100	98.57	97.14	100	100	96.0	92.31	0.98	0.96	[2]	[2]	0.02	0.04
	4.0	95.65	95.65	100	100	97.14	97.14	100	100	92.31	92.31	0.96	0.96	[2]	[2]	0.04	0.04
	4.5	93.48	95.65	100	100	95.71	97.14	100	100	88.89	92.31	0.93	0.96	[2]	[2]	0.07	0.04
	5.0	86.96	95.65	100	100	91.43	97.14	100	100	80	92.31	0.87	0.96	[2]	[2]	0.13	0.04
	5.5	84.78	95.65	100	100	90	97.14	100	100	77.42	92.31	0.85	0.96	[2]	[2]	0.15	0.04
20	0.5	100	97.83	66.67	62.5	88.57	85.71	85.19	83.33	100	93.75	0.67	0.6	3.00	2.61	[1]	0.03
	1.0	100	97.83	95.83	75	98.57	90	97.87	88.24	100	94.74	0.96	0.73	24.00	3.91	[1]	0.03
	1.5	100	97.83	100	83.33	100	92.86	100	91.84	100	95.24	1.00	0.81	[2]	5.87	[1]	0.03
	2.0	100	97.83	100	100	100	98.57	100	100	100	96	1.00	0.98	[2]	[2]	[1]	0.02
	2.5	100	95.65	100	100	100	97.14	100	100	100	92.31	1.00	0.96	[2]	[2]	[1]	0.04
	3.0	97.83	95.65	100	100	98.57	97.14	100	100	96	92.31	0.98	0.96	[2]	[2]	0.02	0.04
	3.5	95.65	95.65	100	100	97.14	97.14	100	100	92.31	92.31	0.96	0.96	[2]	[2]	0.04	0.04
	4.0	89.13	95.65	100	100	92.86	97.14	100	100	82.76	92.31	0.89	0.96	[2]	[2]	0.11	0.04
	4.5	84.78	95.65	100	100	90	97.14	100	100	77.42	92.31	0.85	0.96	[2]	[2]	0.15	0.04
	5.0	82.61	95.65	100	100	88.57	97.14	100	100	75	92.31	0.83	0.96	[2]	[2]	0.17	0.04
	5.5	82.61	93.48	100	100	88.57	95.71	100	100	75	88.89	0.83	0.93	[2]	[2]	0.17	0.07
30	0.5	100	97.83	54.17	45.83	84.29	80	80.7	77.59	100	91.67	0.54	0.44	2.18	1.81	[1]	0.05
	1.0	100	97.83	91.67	70.83	97.14	88.57	95.83	86.54	100	94.44	0.92	0.69	12.00	3.35	[1]	0.03
	1.5	100	97.83	100	83.33	100	92.86	100	91.84	100	95.24	1.00	0.81	[2]	5.87	[1]	0.03
	2.0	95.65	97.83	100	91.67	97.14	95.71	100	95.74	92.31	95.65	0.96	0.89	[2]	11.74	0.04	0.02
	2.5	91.3	97.83	100	100	94.29	98.57	100	100	85.71	96	0.91	0.98	[2]	[2]	0.09	0.02
	3.0	84.78	95.65	100	100	90	97.14	100	100	77.42	92.31	0.85	0.96	[2]	[2]	0.15	0.04
	3.5	82.61	95.65	100	100	88.57	97.14	100	100	75	92.31	0.83	0.96	[2]	[2]	0.17	0.04
	4.0	78.26	95.65	100	100	85.71	97.14	100	100	70.59	92.31	0.78	0.96	[2]	[2]	0.22	0.04
	4.5	78.26	95.65	100	100	85.71	97.14	100	100	70.59	92.31	0.78	0.96	[2]	[2]	0.22	0.04
	5.0	76.09	95.65	100	100	84.29	97.14	100	100	68.57	92.31	0.76	0.96	[2]	[2]	0.24	0.04
	5.5	71.74	93.48	100	100	81.43	95.71	100	100	64.86	88.89	0.72	0.93	[2]	[2]	0.28	0.07

DOB: Delta-over-baseline; [1]: ≈ 0; [2]: Φ +.

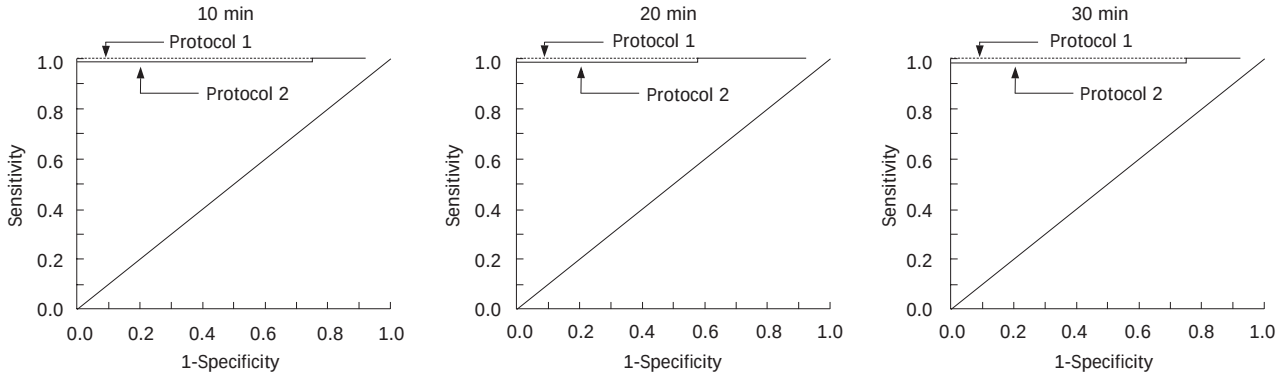

Figure 1 ROC curves for protocols 1 and 2 at 10, 20 and 30 min to establish the diagnosis of *H pylori* infection.

Table 3 shows a selection of 40 studies from the literature where relevant variations to the original ^{13}C-UBT protocol[28] have been implemented. From each study, the protocol with the best diagnostic performance was selected[32-71]. Of these, 12 (30%) yielded sensitivities and specificities of 100%[33,37,43,45,47,56,61,63,65,67-69].

Based on the results after reviewing the literature, the present study introduced several variations to simplify even further the technique and make it more cost-efficient, without compromising the high standards of sensitivity, specificity, positive and negative predictive values of the test. Below is a brief review of the evolution of the assay, since its first description, which led to the designing of the protocols evaluated in the present study.

Urea dose

Originally, the ^{13}C-UBT was described with a dose of ^{13}C-urea of 5 mg/kg of bodyweight[28]. Later on, doses of

Table 2 Distribution of DOB values in *H pylori* positive and negative individuals for both protocols at 10, 20 and 30 min

H pylori-positive individuals						
	10 min		20 min		30 min	
	Protocol 1	Protocol 2	Protocol 1	Protocol 2	Protocol 1	Protocol 2
Mean	17.38	17.69	18.25	22.32	15.44	22.08
Median	13.64	12.02	12.63	17.07	10.98	17.50
SD	14.47	12.68	22.80	15.01	17.75	13.00
H pylori-negative individuals						
	10 min		20 min		30 min	
	Protocol 1	Protocol 2	Protocol 1	Protocol 2	Protocol 1	Protocol 2
Mean	0.32	0.33	0.11	0.45	0.21	0.62
Median	0.51	0.32	0.28	0.34	0.38	0.59
SD	0.91	0.8	0.77	0.86	0.84	0.91

DOB: Delta-over-baseline.

125[35,72] and 100 mg[36,40,47,48,51,54,57,60,62,65,73-83] were validated by several American, European and Asian groups, and more recently 75 mg[32,37,43-45,49,50,55,72,84-93], 50 mg[52,56,58,94,95], 38 mg[96], 25 mg and even 10 mg[69] of ^{13}C-urea have proved to be sufficient.

Test meal

From the beginning, there has been a belief that a delay in gastric emptying is necessary for optimal performance of the test, to allow enough time for the *H pylori*-urease to react in the gastric mucosa, if present. Initially individuals were given a meal consisting of one can of "Sustacal" pudding or 120 mL of 25% glucose polymer, followed 10 min later by a polycose solution containing the ^{13}C-urea[28]. Through the years there have been numerous modifications to the test meal, including the use of citric acid alone before administering the urea[37,62,67,79,84,85,97], or mixed with the ^{13}C-urea at the time of administration[43-45,88,91,98], or as a presentation in combination with the ^{13}C-urea[42,61,72,94,99]. Several alternatives to citric acid have also been tested, including orange juice[43,49,67,90,95] and apple juice[100], as well as other types of food such as milk[48,58,101,102] and a pudding test meal[34,46], and even water[41,103]. As shown in Table 3, the majority of the test meals have provided reliable results. Even the complete absence of a test meal has shown little, if any, variation in the diagnostic performance of the assay[35,40,47,53,56,59,60,102,104].

Via of administration and formulation of ^{13}C-urea

Another issue that has been addressed by different groups is the interference of other urease-producing bacteria in the oral cavity and oropharynx[105,106], leading to an increase in false positive values. As a result, there have been different approaches in the formulation and way of administration of the labeled urea, including the development of ^{13}C-urea tablets[42,61,63,64,95], capsules[68,96,99,107], and even the intragastric instillation of the urea through the endoscope[80,108,109]. Some have also suggested mouth rinsing before and after urea administration[39-41,48,51,52,54,58,60,68,110].

Sample collection times

The ^{13}C-UBT was originally described with a basal sample and 18 post-urea samples taken during the following 180 min[28]. Rapidly the assay was modified and currently only 2 samples are obtained: pre and post-urea. Sampling times, although shorter than initially, have differed among protocols.

Ways of reducing the cost of the ^{13}C-UBT could include decreasing the amount of ^{13}C-urea used, reducing the duration of the test, and improving the ease with which the test can be administered and tolerated. The conventional European ^{13}C-UTB protocol used in our region is sensitive and specific enough (values close to 100%), but it takes 40-45 min to complete and is performed using 100 mg of ^{13}C-urea. For the present study we decided to use 50 mg of ^{13}C-urea to reduce the cost of the assays by half, a dose that has proved to be as accurate as higher doses[52,56,58,61,63,68]. The ^{13}C-urea was administered diluted in 10 mL of water and taken as a single swallow, to try to avoid cross-contamination with urease-producing oropharyngeal bacteria. Immediately after 200 mL of water (pH 6.0) with 4.2 g of citric acid (Protocol 2) and without citric acid (Protocol 1) were administered, and volunteers were asked to make a circular motion around the waistline for a few times to homogenize the aqueous solution and allow contact of the ^{13}C-urea with the entire gastric mucosa. It has been shown that *H pylori* urease operates in a pH range from 3.1 to 10, with an optimal activity at pH 6.0[111,112]. By utilizing water at pH 6.0, activity of the *H pylori* urease was optimized for Protocol 1, where no citric acid was used. Acid solutions have been used by many to delay gastric emptying and to provide a higher acidic environment to induce *H pylori*-urease activity[43,98], although it has been demonstrated by Pantoflickova *et al*[100] that the emptying is determined by the caloric density of the test meal rather than by its pH. Finally, in order to reduce the duration of the test, both protocols were tested at different sampling times: 10, 20 and 30 min.

This study included 70 individuals, 34.3% males and 65.7% females, with an average age of 39.63 ± 12.58 years for males and 34.33 ± 10.17 years for females. According to the reference protocol, 46 (65.7%) individuals were positive for *H pylori* infection. No statistically significant association was found between gender and presence of *H pylori* infection ($P = 0.515$).

There were no significant differences in the ability of both protocols to correctly identify positive and negative *H pylori* individuals at the various sampling times. However, only Protocol 1, with no test meal, yielded a test with sensitivity, specificity, positive and negative predictive values, and accuracy of 100% when compared to the gold standard, when using a DOB cut-off value between 2 and 2.5 at 10 and 20 min, and a DOB cut-off value of 1.5 at 20 and 30 min. For Protocol 2, with citric acid, the highest accuracy (98.57%) was achieved at 10 min using a DOB cut-off value of 2.0, at 20 min a DOB cut-off value of 2.0, and at 30 min with a DOB of 2.5.

Median DOB for *H pylori* infected individuals at 10 min was 13.64, while for Protocol 2 was 12.02. There was no statistically significant difference between these 2 values (Wilcoxon, $P = 0.121$). However, median DOB values at 20 and 30 min for both protocols showed significant differences ($P = 0.006$ and $P = 0.001$, respectively). These results are in accordance with those by Atherton *et al*[113]

Table 3 ^{13}C-UBT protocol with best diagnostic performance from each study with samples obtained within 30 min of ^{13}C-urea administration: Review of literature

First author (reference)	Year	Measuring equipment	Gold standard	*n*	Pre-analytical	^{13}C-urea dose (mg)	^{13}C-urea formulation and via of administration	Test meal	Additional information related to ^{13}C-urea administration	*t*	Cut-off point (DOB)	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)	Acc. (%)
Braden[32]	1994	IRMS	^{13}C-UBT	217	Overnight fasting	75	NA	None		20	5	99	100			
Koletzko[33]	1995	IRMS, NDIRS	^{13}C-UBT	51	Overnight fasting	75	Powder in 150 mL 0.033 mol/L citric acid solution	Taken with ^{13}C-urea		15	5	100	100			
Klein[34]	1996	IRMS	H	465	NA	125	Powder in 90 mL sterile water (Meretek kit)	Ensure		30	2.4	95.4	87.9			94.8
Malaty[35]	1996	IRMS	H, RUT, C	66	Overnight fasting	125	Powder in 100 mL water	None		20	2.4	96	100			
Taniguchi[36]	1996	NDIRS	H	153	Overnight fasting	100	Powder in 30 mL water	None		15	1	97.8	74			
Domínguez-Muños[37]	1997	IRMS	H, RUT, C	80	Overnight fasting	80	Powder in 50 mL water	200 mL 0.1 mol/L citric acid solution		30	4	100	100			
Epple[38]	1997	IRMS	H	77	Overnight fasting	75	NA	Citric acid		30	1.3	96	100			
Kato[39]	1998	IRMS	H, C, RUT	133	Overnight fasting	100	Powder in 100 mL of water	None	Mouth rinsing after ^{13}C-urea	10	3.5	99	100			
Miwa[40]	1998	IRMS	H	409	8 h fasting	100	Powder	None	Mouth rinsing before and after ^{13}C-urea	20	5	97	97			
Ohara[41]	1998	IRMS	H, RUT, C	248	Overnight fasting	100	Powder in 100 mL tap water	None	Mouth rinsing after ^{13}C-urea	20	2.5	98	98			98
Hamlet[42]	1999	IRMS	^{13}C-UBT, H, RUT, C	134	Overnight fasting	100	Two tablets (Diabact UBT) with 50 mg of ^{13}C-urea + 456 mg of anhydrous citric acid swallowed with 200 mL of water	Taken with ^{13}C-urea		10	1.8	95	100			
Leodolter[43]	1999	IRMS	H, RUT, C	50	NA	75	Powder in 200 mL 0.1 mol/L citric acid solution	Taken with ^{13}C-urea		30	4	100	100			
Leodolter[44]	1999	IRMS	H, RUT, C	233	NA	75	Powder in 200 mL citric acid solution	Taken with ^{13}C-urea		30	4	95	98			97
Savarino[45]	1999	IRMS	H, RUT	134	Overnight fasting	75	Powder in 150 mL 0.033 mol/L citric acid solution	Taken with ^{13}C-urea		30	5	100	100			
Van der Hulst[46]	1999	LARA	H, C	544	NA	100	Powder in 50 mL sterile water	Ensure		30	6.3-7.5	93-95	94-96	95-98	86-94	
Gisbert[47]	2000	IRMS	^{13}C-UBT, H	53	Overnight fasting	100	Powder in 50 mL water (TAU-KIT)	None		30	3.3-3.9	100	100			
Peng[48]	2000	IRMS	H, RUT, C	136	6 h fasting	100	Powder in 50 mL sterile water	100 mL milk	Mouth rinsing after ^{13}C-urea and laid on their sides, changing sides every 5 min	15	4.8	94	89			
Riepl[49]	2000	NDIRS	H, C	100	Overnight fasting	75	Powder in 200 mL orange juice	Taken with ^{13}C-urea		15	6.5	92	94	89	94	

Savarino[50]	2000	IRMS	H, RUT	117	Overnight fasting	75	Powder in 150 mL 0.033 mol/L citric acid solution	Taken with ^{13}C-urea		30	5	98	97	98	97	98
Sheu[51]	2000	IRMS	H, C	441	Overnight fasting	100	NA	100 mL of fatty test meal	Mouth rinsing before and after ^{13}C-urea	15	4	98	97			
Sheu[52]	2000	IRMS, NDIRS	H, C	177	Overnight fasting	50	NA	100 mL citric acid solution	Mouth rinsing after ^{13}C-urea	15	3.5	96	99	99	97	
Wong[53]	2000	IRMS	H, RUT	202	Overnight fasting	75	Powder in 50 mL distilled water	2.4 g of citric acid		30	5	96	98	98	96	97
Yoshida[54]	2000	LARA	H, C, PCR	104	Overnight fasting	100	Powder in 50 mL distilled water	None	Mouth rinsing after ^{13}C-urea	20	2.7	98	100			99
Mana[55]	2001	NDIRS	H	223	Overnight fasting	75	Powder	20 mL 0.1 mol/L citric acid solution		10		100	95	94	100	
Wong[56]	2001	IRMS	H, RUT	101	Overnight fasting	75	Powder in 50 mL water	None		30	3.5-4.5	100	100			100
Chua[57]	2002	IRMS	H, RUT, S	100	NA	100	Powder in solution containing citric acid and ^{13}C-urea		Pacients laid on their left side for 30 min	30	3.5	94	100	100	89	
Liao[58]	2002	IRMS	H, RUT	152	Overnight fasting	50	Powder in 50 mL sterile water	200 mL full-cream cow's milk	Patients gargled with water 3 times after ^{13}C-urea and laid on their sides, changing sides every 3 min	15	2.5-3.0	99	97	99	97	99
Ng[59]	2002	IRMS	H, RUT	123	Regular meal within 2 h of the ^{13}C-urea	75	Powder in 50 mL water	2.4 g citric acid in 200 mL solution		30	5.5	93	97	100	97	
Chen[60]	2003	NDIRS	H, RUT, C, SAT	586	Overnight fasting	100	Powder in 100 mL of water	None	Patients gargled with water 3 times after ^{13}C-urea and laid down on the left side for 5 min	20	3.5	98	97			98
Gatta[61]	2003	IRMS	H, RUT, C	200	Overnight fasting	50	Tablet (Diabact UBT) with 50 mg of ^{13}C-urea and 456 mg of anhydrous citric acid swallowed with 200 mL of water	Citric acid		10	1.65-3.15	100	100			
Gisbert[62]	2003	IRMS	H, RUT	36	Overnight fasting	100	Powder in 50 mL water (TAU-KIT)	200 mL solution with 4.2 g citric acid		30	5	96	100	100	91	
Wong[63]	2003	IRMS	H, RUT	150	Overnight fasting	50	Tablet (Diabact UBT) with 50 mg of ^{13}C-urea and 456 mg of anhydrous citric acid swallowed with 200 mL of water	Citric acid		20	2.1	100	100			
Ohara[64]	2004	IRMS	^{13}C-UBT, H, C, RUT	254	Overnight fasting	100	Film-coated tablet swallowed with 100 mL of water	None		20	2.5	98	98			98

Urita[65]	2004	IRMS	H, S	129	Overnight fasting	100	Powder in 100 mL tap water	None	Sample taken through nostril	20	2.5	100	100			100
Beiki[66]	2005	NDIRS	^{14}C-UBT, H, RUT	76	Overnight fasting	75	Powder in 200 mL orange juice			30	3.5	100	97	98	100	99
Kopacova[67]	2005	IRMS	^{13}C-UBT	27	Overnight fasting	100	Powder in 50 mL distilled water with 1 g citric acid	150 mL distilled water with 3 g citric acid, orange juice or distilled water		10	3.5	100	100			100
Peng[68]	2005	IRMS	H, RUT, C	50	6 h fasting	100	Capsule with water	None	Mouth rinsing before and after ^{13}C-urea and laid on their sides, changing sides every 5 min	15	4-5	100	100			100
Gatta[69]	2006	IRMS	H, RUT	100	Overnight fasting	25	Dissolved in water	Citric acid (1 g)		30	4.4-6.26	100	100			
Mauro[70]	2006	IRMS	H, C	176	Overnight fasting	75	Powder in 100 mL citric acid solution	Taken with ^{13}C-urea		30	3	100	99	95-98	100	
Mauro[71]	2006	IRMS	H, C	67	Overnight fasting	75	Powder in 100 mL citric acid solution	Taken with ^{13}C-urea		10	3	100	96	95-98	99-100	
Present study	2007	IRMS	^{13}C-UBT	70	Overnight fasting	50	Powder in 10 mL sterile water immediately followed by 200 mL sterile water	None	Patients made a circular motion around the waistline for a few times	10	2.0-2.5	100	100	100	100	100

n: Participating individuals; *t*: Sampling time; PPV: Positive predictive value; NPV: Negative predictive value; Acc: Accuracy; UBT: Urea breath test; H: histology; C: Culture; RUT: Rapid urease test; S: Serology; NA: Not available; IRMS: Isotope ratio mass spectrometry; NDIRS: Non-dispersive infrared spectrometry; LARA: Laser assisted ratio analyser; DOB: Delta-over-baseline.

and Gisbert *et al*[47], who showed that the test meal did not affect ^{13}C-UBT results at 10 min, but increased the values thereafter.

In conclusion, an optimal laboratory test should be non-invasive, easy to perform, highly reproducible, cost-efficient and with a sensitivity and specificity close to 100%. When compared to other protocols published in the literature, the present conditions of Protocol 1 have further optimized the ^{13}C-UBT assay, as this is the only protocol with a sampling time of 10 min, a ^{13}C-urea dose of 50 mg and no test meal that can yield a test with 100% accuracy for the diagnosis of *H pylori* infection. These variations provide a protocol that can reduce the cost of the ^{13}C-UBT assay, is innocuous, well tolerated, has no restrictions and could be implemented for all patients in whom endoscopy is not an indication[21,22] and as a screening test for *H pylori* epidemiological studies. Further studies are underway to try to decrease the ^{13}C-urea to an even lower dose, using biopsy as the gold standard.

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COMMENTS

Background

H pylori infection is present in around 50% of the world population and has been associated with the pathogenesis of gastric disorders such as gastritis, gastric ulcer and MALT lymphoma, and a variety of extradigestive diseases, including idiopathic thrombocytopenic purpura, iron deficiency anemia and autoimmune thyroiditis, among others. Diagnosis of *H pylori* infection can be established by either invasive techniques, by means of endoscopy, or non-invasive techniques such as the ^{13}C-urea breath test.

Research frontiers

The ^{13}C-urea breath test relies upon the ability of an enzyme (urease), produced by *H pylori* in the stomach, to break down the administered urea. Patients swallow the urea labelled with a non-radioactive isotope (^{13}C). After a few minutes, the isotope-labelled carbon dioxide ($^{13}CO_2$) is exhaled in the breath if there is presence of *H pylori* urease in the stomach. The difference in the $^{13}CO_2$ values before and after ingestion of the labelled urea will determine the presence of infection.

Innovations and breakthroughs

Many have attempted to lower the high cost of the ^{13}C-urea breath test, while preserving excellent diagnostic accuracy. For this purpose, modifications in the

dose, formulation and way of administration, sample collection times and test meals have been evaluated.

Applications

A low cost ^{13}C-urea breath test for the detection of *H pylori* infection before and after eradication treatment will make this non-invasive assay more accessible for patients, especially in developing countries.

Terminology

^{13}C-UBT: Breath test that includes urea labelled with ^{13}C, a non-radioactive isotope. DOB: Delta over base line, units used to express the amount of $^{13}CO_2$ contained in the breath sample.

Peer review

This is a well written and comprehensively referenced article. The methods section is adequately described and the results clearly presented. The conclusions are a fair interpretation of the results.

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RAPID COMMUNICATION

Improved survival for hepatocellular carcinoma with portal vein tumor thrombosis treated by intra-arterial chemotherapy combining etoposide, carboplatin, epirubicin and pharmacokinetic modulating chemotherapy by 5-FU and enteric-coated tegafur/uracil: A pilot study

Toru Ishikawa, Michitaka Imai, Hiroteru Kamimura, Atsunori Tsuchiya, Tadayuki Togashi, Kouji Watanabe, Kei-ichi Seki, Hironobu Ohta, Toshiaki Yoshida, Tomoteru Kamimura

Toru Ishikawa, Michitaka Imai, Hiroteru Kamimura, Atsunori Tsuchiya, Tadayuki Togashi, Kouji Watanabe, Kei-ichi Seki, Hironobu Ohta, Toshiaki Yoshida, Tomoteru Kamimura, Department of Gastroenterology and Hepatology, Saiseikai Niigata Second Hospital, Niigata 950-1104, Japan
Correspondence to: Toru Ishikawa, MD, Department of Gastroenterology and Hepatology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata 950-1104, Japan. toruishi@ngt.saiseikai.or.jp
Telephone: +81-25-2336161 Fax: +81-25-2338880
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Abstract

AIM: To investigate the poor prognosis of HCC with PVTT, we evaluated the efficacy by a new combination chemotherapy for advanced hepatocellular carcinoma (HCC) with portal vein tumor thrombus (PVTT).

METHODS: From 2002 to 2007, a total of 10 consecutive patients with Stage IVA HCC accompanied by PVTT were studied prospectively to examine the efficacy of treatment by intra-arterial infusion of a chemotherapeutic agents consisting of etoposide, carboplatin, epirubicin and pharmacokinetic modulating chemotherapy by 5-FU and enteric-coated tegafur/uracil.

RESULTS: The mean course of chemotherapy was 14.4 (range, 9-21) mo. One patient showed complete response (CR) with disappearance of HCC and PVTT after treatment, and the two patients showed partial response (PR), response rate (CR + PR/All cases 30%). The median survival time after the therapy was 457.2 d. The one-year survival rate was 70%. Adverse reactions were tolerable.

CONCLUSION: Although the prognosis of most patients with Stage IVA HCC by PVTT is poor, our combination chemotherapy may induces long-term survival and is an effective treatment and produced anti-tumor activity with tolerable adverse effects in patients for advanced Stage IVA HCC accompanied by PVTT.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide including Japan[1]. Although the development of imaging modalities has made the early diagnosis of HCC possible, surgically resectable cases are relatively uncommon because of a hepatic function reserve and/or an advanced stage at presentation. Several modalities, such as percutaneous ethanol injection (PEI), transcatheter arterial embolization (TAE), chemolipiodolization, microwave coagulation therapy (MCT), and radiofrequency ablation therapy (RFA) are reportedly useful in treating patients with unresectable disease[2,3]. However, unfortunately, many hepatocellular carcinoma patients have tumor recurrence. Furthermore, HCC has a predilection for portal vein invasion, which has been shown to be a poor prognostic factor. An effective therapy regimen is needed for advanced HCC with portal vein tumor thrombus (PVTT). Recent trials have been reported that combination therapy of intra-arterial 5-FU and systemic interferon for HCC with PVTT is effective[4,5]. However, portal venous invasion is a crucial factor that can worsen the prognosis of patients with HCC. It often leads to extensive spreading of the tumor throughout the liver, and can increase portal venous blood pressure, resulting in the fatal rupture of esophageal varices, and can decrease portal flow which causes ascites, jaundice, hepatic encephalopathy, and liver failure. Previous studies have

reported that the median survival time of patients with portal venous invasion was 2.7-4 mo if left untreated[6,7].

As systemic therapy of HCC, we had previously achieved complete remission of multiple HCC associated with hepatitis C virus-related decompensated liver cirrhosis by oral administration of enteric-coated tegafur/uracil[8]. Furthermore, we reported that oral administration of enteric-coated tegafur/uracil induces long-term survival and is an effective treatment for Stage Ⅳ-A HCC[9]. However, this therapy by single agent is not effective HCC with PVTT. Therefore, there is an urgent need for new method and active drugs for PVTT from HCC.

The aim of this study is to evaluate the usefulness by intra-arterial infusion of a chemotherapeutic agents consisting of etoposide, carboplatin, epirubicin and pharmacokinetic modulating chemotherapy by 5-FU and enteric-coated tegafur/uracil for HCC with tumor thrombosis of the main trunk of the portal vein.

MATERIALS AND METHODS

Ethics

The study protocol was reviewed and approved by the Hospital Ethics Committee. Informed consent was obtained from each patient who entered a randomized controlled trial and from family member(s).

Patients

A group of 10 consecutive patients with HCC accompanied by portal vein tumor thrombus (PVTT) were enrolled in the therapeutic trial between April 2002 and April 2007. The diagnosis of HCC was made by histologically and/or imaging study.

All patients received intra-arterial regional chemotherapy carried out at the Department of Gastroenterology and Hepatology, Saiseikai Niigata Second Hospital and gave their informed consent according to our situational guidelines, and the study received ethical approval.

Eligibility criteria for patients in this study included the following: (1) diagnosed Stage ⅣA HCC with PVTT (2) unresectable carefully assessed by the individual experts; (3) no recent active treatments including surgery, radiotherapy, chemotherapy, transarterial embolization, percutaneous ethanol injection, or other regional treatment within six month; (4) HCC with PVTT diagnosed by total image systems such as computed tomography (CT) or magnetic resonance imaging (MRI). (5) the ability to manage the indwelling catheters and implanted injection ports; (6) adequate hematologic function (white blood cell count > 3000/L, platelet count > 80 000/L, and hemoglobin level > 9.5 gm/dL); adequate renal function (serum creatinine < 1.5 mg/dL and a creatinine clearance > 60 mL/min); (7) adequate hepatic function, (8) a performance status less than 3 at pre-treatment, (9) portal tumor thrombi located the first portal branch, or the main portal trunk, and (10) informed consent.

Treatment schedule and follow up

For intra-arterial regional chemotherapy, catheters were introduced into the proper or common hepatic artery placed *via* the right femoral artery using the Seldinger method. The gastroduodenal artery and the right artery were occluded by a steel coil as indicated to prevent gastroduodenal injury from the anticancer agents. Intra-arterial regional chemotherapy was performed by puncturing a thin needle percutaneously into the port. Before every infusion, we confirmed that the catheters were patent, either by checking the blood back-flowing in the catheter or by injecting a contrast medium under fluoroscopy. Using an infusion pump (Syringe Pump, Terumo Co. Ltd., Osaka, Japan), 50 mg/body of etoposide (VePesid, Bristol-Myers Squibb Co. Ltd., Tokyo, Japan), 300 mg/body of carboplatin (Paraplatin, Bristol-Myers Squibb Co. Ltd., Tokyo, Japan) and 60 mg/body of epirubicin (Farmorubicin, Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) were infused from each catheter over a 30-minute period. Subsequently, a continuous arterial infusion of 5-FU (500 mg/m^2) (5-FU, Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was given for 24 h. Before the needle was removed from the port, both the port and connected catheter were filled with undiluted heparin (1000 U/mL). An antiemetic and an H2-receptor antagonist were given intravenously. This treatment was repeated once weekly for 3 consecutive weeks of every 4 wk or biweekly, mainly at our outpatient clinic, for as long as possible. All patients were given also enteric-coated tegafur/uracil (Taiho Pharmaceutical, Co. Ltd., Tokyo, Japan) at a dose rate of 200 mg/body twice daily. Treatment was continued for a minimum of 8 wk and given until patient withdrawal or death even after the progression of disease.

Studies during follow-up included a physical examination, complete blood count, platelet count, and determination of levels of AFP, PIVKA-Ⅱ, amylase, liver transaminase, urea nitrogen, and creatinine. Either an ultrasonogram or a computed tomogram of the abdomen was obtained at least every two months, to determine the size of HCC and PVTT.

Evaluation of therapeutic response

The therapeutic clinical response of the liver was assessed in accordance with the World Health Organization Criteria. Clinical responses were graded as follows. Complete response (CR) was defined as disappearance of all measurable lesions in the liver, continuing for at least 4 weeks when assessed by both computed tomography and ultrasonography. Reduction of tumor size by more than 50%, continuing for at least 4 wk, was regarded as partial response (PR). No change (NC) was determined as tumors showing a decrease in size of less than 25%. Progressive disease (PD) was defined as tumors that had grown over 25%.

Statistical analysis

Survival curves were calculated by the Kaplan-Meier method and the difference between survival curves was evaluated using the log-rank test.

Statistical analyses were performed using Stat View-J4.11 software (Abacus Concepts; Berkeley, California) to assess the relative prognostic importance

Table 1 Characteristics of the patients

10 patients
Age mean 60.20 y.o (range 35-70)
Sex: Male/Female 7/3
Child-Pugh's stage: A,B/C 8/2
"Virus: HBsAg/anti-HCV 8/2"

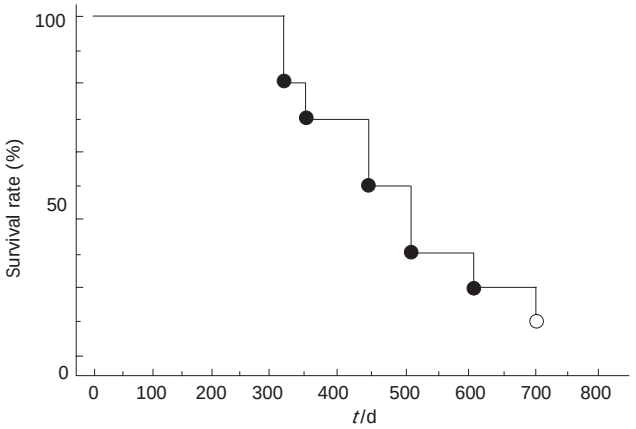

Figure 1 Cumulative survival of patients with hepatocellular carcinoma accompanied by PVTT who treated combination therapy.

of variables in predicting the survival rate. Differences at $P < 0.05$ were considered significant.

RESULTS

Background clinical and laboratory data of patients

Patients' profiles before the combination chemotherapy are listed in Table 1. Seven men and three women, with a median age of 60.2 years, were treated. Positive HBsAg was found in 70% of patients, while anti-hepatitis C virus (HCV) serology was positive in 30% of patients.

Child-Pugh Grade A/B was 2/6, the remaining patients had Grade C. Only 2 patients received the full courses of chemotherapy. The median number of cycles received per patients was 15.3. Dose reduction was required in 80% of the patients, mainly due to profound bone marrow suppression from the previous cycle.

Cumulative survival rate of patients by Kaplan-Meier survival curves

Of the 10 patients in the treated group, a total of 3 (30.0%) were classed as CR or PR, and a total 7 (70.0%) were classed as NC or PD. At the time of the final analysis (April 2007) 2 patients (from the treatment group) were alive. A total of 153 treatment cycles were administered.

The overall survival curve for all 10 patients is shown in Figure 1. The median survival was 457.2 d. The survival rates at the end of 1-year and 2-year were 70.0% and 20.0%, respectively. Of the 10 patients studied, 8 had died by the time of this analysis. The survival curves for clinical responders (CR or PR) and the others (NC or PD) are shown in Figure 2. The 6-mo and one-year survival rates were 100% and 100.0%, respectively, in the responders

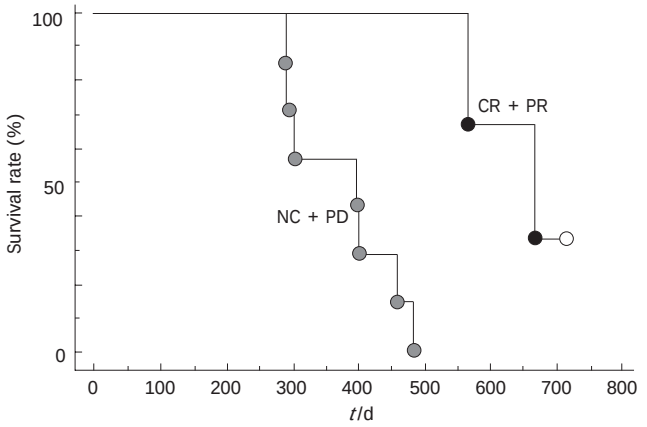

Figure 2 Survival of patients with hepatocellular carcinoma accompanied by PVTT according to the response and control (*Log-rank P < 0.05)*. CR: Complete response, PR**;** Partial response, NC; No change, PD; Progressive disease.

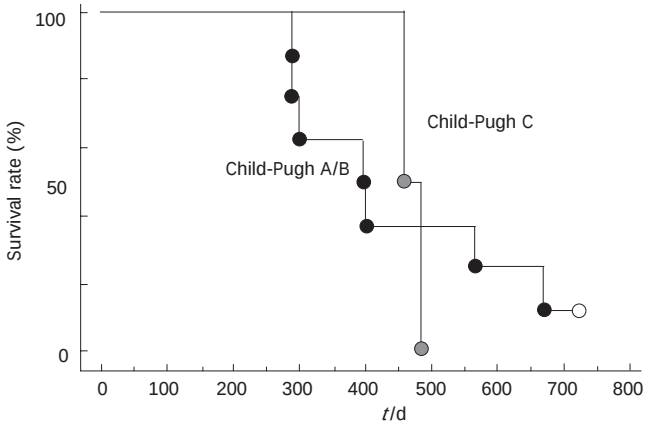

Figure 3 Survival of patients with hepatocellular carcinoma accompanied by PVTT according to Child-Pugh's stage (*Log-rank P = 0.98)*.

and 100% and 57.1%, respectively, in the non-responder. There was a significant difference in survival between the two groups ($P < 0.01$).

However, there was not a significant difference in survival rates between Child-Pugh A/B group and Child-Pugh C group (Figure 3).

Side effects and complications due to regional chemotherapy

The side-effects and complications encountered during therapy are summarized in Table 2. Local complications at the femoral artery entry sites did not occur in any patient. No serious complications that necessitated intensive care were encountered during therapy. The side-effects included oral dryness, diarrhea and liver dysfunction, and bone marrow suppression. Mild oral dryness was noted at the beginning of the treatment in 40.0% of patients, but this subsided as treatment continued. Mild diarrhea was noted at the beginning of the treatment in 30.0% of patients, but this also subsided with time. Such symptoms resolved spontaneously or after appropriate therapy. The complications experienced by patients treated with regional chemotherapy were well tolerated. No other serious

Table 2 Main clinical side effects observed during the treatment (*n* represents the number of patients having experienceed the effect in any of the courses)

	Grade of toxicity			
	1	2	3	4
Oral dryness	3	1	0	0
Diarrhea	1	2	0	0
Vomiting	1	0	0	0
Liver dysfunction	1	2	0	0
Fever	0	0	0	0
Hair loss	1	0	0	0
BM suppression	4	4	0	0

complications, such as gastric ulcer, liver damage, renal damage, vascular complications, or cardiac toxicity were encountered. However, dose reduction was required in 80% of the patients, mainly due to profound bone marrow suppression from the previous cycle. There were no treatment-related deaths from administration of regional chemotherapy.

DISCUSSION

Patients with HCC are highly compromised by failing liver function. HCC is associated with a high risk of portal vein involvement. PVTT is one of important prognostic factors in patients with HCC. However, HCC with PVTT is refractory to treatment. The treatment of HCC with PVTT is still problematic and major challenge item for oncologists because of its high and dismal outcomes. None of the reported treatment regimens can be considered to be standard treatment for HCC with PVTT.

Meanwhile, regional hepatic arterial infusion chemotherapy is a reasonable drug delivery system for patients with advanced HCC because the tumors derive most of their blood supply from the hepatic artery, whereas the portal vein supplies the normal parenchyma[10]. Furthermore, chemotherapy combined with interferon is reported to be effective for HCC with PVTT.

Combined treatment with 5-FU and alpha-interferon for HCC patients was first reported by Patt, *et al* in 1993[11]. The response rate was reportedly 22%. Urabe *et al*[12] treated 16 patients with HCC and PVTT in the main trunk or the major branches of the portal vein by intrahepatic infusion of methotrexate, 5-FU, and cisplatin, and administered alpha-interferon subcutaneously. The response rate and median survival time were 46.7% and 7 mo, respectively.

Moreover, combined intra-arterial 5-FU and subcutaneous alpha-interferon therapy for 8 patients with HCC accompanied by PVTT in the major portal vein was reported by Sakon *et al*[5]. The response rate was 63%. In another study by this group in 2005, 55 patients received this treatment, and 8 (14.5%) showed a complete response, 16 (29.1%) showed a partial response, 4 (7.3%) showed no response, and 27 (49.1%) showed progressive disease[13]. The median survival time and 5-year survival rate were 11.8 mo and 16.4%, respectively. Using this combination protocol, Obi *et al*[4] treated 116 patients with unresectable HCC accompanied by PVTT in the main trunk or the 1st branches of the portal vein. The survival rates at 1 and 2 years among overall patients were 34% and 18%, respectively, in contrast to 15% and 5% among the historical controls. Survival rates at 1 and 2 years were 81% and 59% among complete responders, respectively, and 43% and 18% among partial responders. The median survival time was prolonged to 11 mo in patients with an active response, although there appears to be no benefit in patients without an active response.

However, it is chaotic to determine whether the combination chemotherapy with interferon is effective or not for HCC accompanied by PVTT.

We previously reported[8,9] that administration of enteric-coated tegafur/uracil induces long-term survival and is an effective treatment for Stage Ⅳ-A HCC. However, single agent such as enteric-coated tegafur/uracil was not effective for HCC with PVTT. So, combination chemotherapy is needed for HCC with PVTT.

The choice of the anticancer agent is important in achieving favorable clinical results. We selected etoposide, carboplatin, epirubicin and pharmacokinetic modulating chemotherapy by 5-FU and enteric-coated tegafur/uracil.

Firstly, etoposide is agent which has shown significant antitumor against HCC[14-16]. It could be suggested as part of intensive multidrug regimens for HCC and high-risk HBV[17-19]. Though response rates to cisplatin and etoposide[20] given systemically as single agents are 5 and 15%, intra-arterial combination chemotherapy using cisplatin and etoposide produces a high rate of objective tumor remissions in patients with HCC[21].

However, cisplatin has been reported that it has a lot of nephrotoxic and emetic effects. To the contrary, carboplatin has demonstrated antitumor activity comparable to cisplatin and has been shown to have fewer nephrotoxic and emetic effects. In fact, carboplatin is thought to be a useful anticancer agent in patients with HCC treated with TACE. Furthermore, it is reported that carboplatin is effective for HCC[22-24].

So we selected carboplatin as combination with etoposide. In addition, a combination of epirubicin and etoposide appears to be an active and tolerable therapeutic option for HCC patients who are not candidates for surgical or locoregional procedures[19,25-27].

Recently, Kusunoki *et al* reported that pharmacokinetic modulating chemotherapy, based on the concept that the benefit of a continuous venous 5-fluorouracil infusion can be potentiated by low-dose oral tegafur/uracil is useful for a variety of cancers[28,29].

In fact, it is reported that modified pharmacokinetic modulating chemotherapy had no severe side effect and was effective for advanced unresectable HCC[30].

Based on these facts, we tried combination chemotherapy for HCC with PVTT. In our series, the treatment resulted in an objective response rate of 30% and a median survival of 457.2 d. Only the three patients who had an objective response had a survival of long duration.

As our group was small, we did not perform a statistical analysis to determine a predictive factor for response. However, our results are comparable with those of most interferon combination chemotherapy.

In our study, the toxicity of this therapy was low despite the fact that all of the patients had cirrhosis. It is noteworthy that there were no patients showing overt liver toxicity. Moreover, there was no treatment-related death.

In this study, no hepatotoxicity due to this combination chemotherapy was observed. The side effects of this regimen were minimal and well tolerated.

In conclusion, this chemotherapeutic regimen ameliorated the survival of patients with advanced HCC without serious adverse effects.

We suggest that, in the near future, this chemotherapy method should be subjected to a prospective randomized controlled study for HCC with PVTT. Further prospective randomized clinical trials of chemotherapy for HCC with PVTT will be needed.

COMMENTS

Background

Portal venous tumor thrombus (PVTT) is a crucial factor that can worsen the prognosis of patients with hepatocellular carcinoma (HCC). It often leads to extensive spreading of the tumor throughout the liver, and can increase portal venous blood pressure, resulting in the fatal rupture of esophageal varices, and can decrease portal flow which causes ascites, jaundice, hepatic encephalopathy, and liver failure. However, there is not effective useful therapy for HCC combined with PVTT. Therefore, there is an urgent need for new and active drugs and combination chemotherapy of advanced HCC.

Research frontiers

HCC has a predilection for portal vein invasion, which has been shown to be a poor prognostic factor. An effective therapy regimen is needed for advanced HCC with PVTT. Combination chemotherapy is needed for HCC with PVTT urgently. The choice of the anticancer agent is important in achieving favorable clinical results. The authors selected etoposide, carboplatin, epirubicin and pharmacokinetic modulating chemotherapy by 5-FU and enteric-coated tegafur/uracil. Progress in implantable drug delivery systems has made possible the repeated arterial infusion of chemotherapeutic agents for patients with advanced HCC recently. Therefore, hepatic arterial infusion chemotherapy has been often selected as a therapeutic option for advanced HCC with PVTT.

Innovations and breakthroughs

The authors investigated the efficacy, the feasibility, usefulness, and complication rate of arterial combination therapies for HCC with PVTT.

Applications

Intra-arterial combination chemotherapy is useful and inducing long-term survival for advanced HCC accompanied by PVTT. Further prospective randomized clinical trials of chemotherapy for HCC with PVTT will be needed.

Terminology

PVTT: Portal vein tumor thrombus meaning tumor thrombus locating the first portal branch, or the main portal trunk.

Peer review

This is an interesting manuscript reporting the strategy for HCC with PVTT. This new information is certainly worthy of publication.

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RAPID COMMUNICATION

Pre- and postoperative systemic hemodynamic evaluation in patients subjected to esophagogastric devascularization plus splenectomy and distal splenorenal shunt: A comparative study in schistomomal portal hypertension

Roberto de Cleva, Paulo Herman, Luis Augusto Carneiro D'albuquerque, Vincenzo Pugliese, Orlando Luis Santarem, William Abrão Saad

Roberto de Cleva, Paulo Herman, Luis Augusto Carneiro D' albuquerque, Vincenzo Pugliese, Orlando Luis Santarem, William Abrão Saad, Gastroenterology Department, University of São Paulo Medical School, São Paulo, SP 05403-900, Brazil
Correspondence to: Roberto de Cleva, Gastroenterology Department, University of São Paulo Medical School, Rua Cel. Artur Godoy 125, Apto 152. Vila Mariana, São Paulo, SP 04018-050, Brazil. roberto.cleva@hcnet.usp.br
Telephone: +55-11-32842831 Fax: +55-11-32842831
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Abstract

AIM: To investigate the systemic hemodynamic effects of two surgical procedures largely employed for treatment of schistosomal portal hypertension.

METHODS: Thirty-six patients undergoing elective surgical treatment of portal hypertension due to hepatosplenic mansonic schistosomiasis were prospectively evaluated. All patients were subjected to preoperative pulmonary artery catheterization; 17 were submitted to esophagogastric devascularization and splenectomy (EGDS) and 19 to distal splenorenal shunt (DSRS). The systemic hemodynamic assessment was repeated 4 d after the surgical procedure.

RESULTS: Preoperative evaluation revealed (mean ± SD) an increased cardiac index (4.78 ± 1.13 L/min per m^2), associated with a reduction in systemic vascular resistance index (1457 ± 380.7 dynes.s/cm^5.m^2). The mean pulmonary artery pressure (18 ± 5.1 mmHg) as well as the right atrial pressure (7.9 ± 2.5 mmHg) were increased, while the pulmonary vascular resistance index (133 ± 62 dynes.s/cm^5.m^2) was decreased. Four days after EGDS, a significant reduction in cardiac index (3.80 ± 0.4 L/min per m^2, $P < 0.001$) and increase in systemic vascular resistance index (1901.4 ± 330.2 dynes.s/cm^5.m^2, $P < 0.001$) toward normal levels were observed. There was also a significant reduction in pulmonary artery pressure (12.65 ± 4.7 mmHg, $P < 0.001$) and no significant changes in the pulmonary vascular resistance index (141.6 ± 102.9 dynes.s/cm^5.m^2). Four days after DSRS, a non-significant increase in cardiac index (5.2 ± 0.76 L/min per m^2) and systemic vascular resistance index (1389 ± 311 dynes.s/cm^5.m^2) was observed. There was also a non-significant increase in pulmonary artery pressure (19.84 ± 5.2 mmHg), right cardiac work index (1.38 ± 0.4 kg.m/m^2) and right ventricular systolic work index (16.3 ± 6.3 g.m/m^2), without significant changes in the pulmonary vascular resistance index (139.7 ± 67.8 dynes.s/cm^5.m^2).

CONCLUSION: The hyperdynamic circulatory state observed in mansonic schistosomiasis was corrected by EGDS, but was maintained in patients who underwent DSRS. Similarly, the elevated mean pulmonary artery pressure was corrected after EGDS and maintained after DSRS. EGDS seems to be the most physiologic surgery for patients with schistosomal portal hypertension.

Key words: Pulmonary Hypertension; Hyperdynamic circulation; Portal Hypertension; Splenectomy; Cardiomyopathy

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INTRODUCTION

In Brazil, mansonic schistosomiasis is an endemic disease, and its hepatosplenic form, which is characterized by presinusoidal portal hypertension with preserved liver function and marked splenomegaly, is a major cause of portal hypertension[1-3]. Upper digestive tract hemorrhage due to esophageal varices rupture is the most feared complication[4].

The development of portal hypertension, regardless of its etiology, is a consequence of increased vascular resistance, mostly due to an architectural distortion of

the liver parenchyma secondary to fibrosis, but also due to a diminished endothelial nitric oxide release from the hepatic endothelium[5]. Increased portal venous inflow due to mesenteric arteriolar vasodilatation, and determined by increased levels of vasodilators, also contributes to portal pressure increase[6].

The pathophysiology of portal hypertension in hepatosplenic mansonic schistosomiasis also displays a systemic hyperdynamic state[2]. We have previously reported that this hyperdynamic circulatory state seems to be corrected in the intraoperative period during esophagogastric devascularization and splenectomy (EGDS)[7]. However, to the best of our knowledge, there are no data in the literature regarding postoperative systemic hemodynamics after surgical treatment of schistosomal portal hypertension.

The purpose of this study was to prospectively investigate the postoperative systemic hemodynamic effects in two different surgical procedures largely employed for treatment of schistosomal portal hypertension.

MATERIALS AND METHODS

Thirty-six patients with portal hypertension and a history of previous upper digestive tract bleeding, due to esophageal varices rupture secondary to hepatosplenic mansonic schistosomiasis, were prospectively studied before and after elective surgical treatment between June 1998 and March 2005. Eighteen patients were male and eighteen female, with a mean age of 39 (range 22-56) years. Laboratory data and arterial blood gases are expressed in Table 1. Transthoracic echocardiography was performed in all patients before surgery. The Hospital Ethics Committee approved the study protocol, and all patients signed their informed consent. Immediately before surgery, patients underwent a right internal jugular vein puncture with the introduction of a pulmonary artery catheter (Edwards Swan-Ganz TM, caliber 7F, model 93A-131H; Baxter Corporation, USA) for invasive systemic hemodynamic assessment. Patients were randomized for two different elective surgical procedures: 17 were subjected to esophagogastric devascularization and splenectomy (EGDS) and 19 to distal splenorenal shunt (DSRS). A mean pulmonary artery pressure greater than 25 mmHg was considered as an absolute contraindication for DSRS.

EGDS consisted of ligation of the splenic artery close to the body of the pancreas, followed by splenectomy and devascularization of the distal 5-7 cm of the esophagus, and of the upper two thirds of the stomach proximal to the incisura angularis. DSRS consisted of dissection of the splenic vein from the splenic hilum until its junction with superior mesenteric vein, and ligating all small vessels between the pancreas and the splenic vein (splenopancreatic disconnection). Left renal vein anterior and superior surfaces were dissected, the splenic vein was transected near it's junction with the superior mesenteric vein, and an anastomosis between the splenic and renal veins was performed with a running suture. No immediate complications were observed and there was no intra- or postoperative mortality.

Four days after surgery, when the surgical effects had worn off, the systemic hemodynamic assessment was repeated and the pulmonary artery catheter was removed. There were no complications related to pulmonary artery catheterization.

Table 1 Preoperative laboratory and arterial blood gases data

	Mean ± SD	Normal values
ALT (IU/L)	31.7 ± 16.8	7-45
AST (IU/L)	31.6 ± 20.5	7-45
Gamma GT (IU/L)	46.2 ± 25.5	7-50
ALP (IU/L)	118.5 ± 46.3	60-122
BUN (mg/dL)	25.5 ± 5.75	10-50
Cr (mg/dL)	0.78 ± 0.16	0.6-1.4
TP (g/dL)	7.45 ± 0.71	6-8
ALB (g/dL)	4.18 ± 0.48	3.5-5.0
PT (s)	14.2 ± 2.9	14 ± 2
PTT (s)	29.8 ± 6.26	30 ± 2
TBIL (mg%)	1.16 ± 0.73	1.4
IBIL (mg%)	0.79 ± 0.67	0.8
Hb (g/dL)	11.2 ± 2.4	12-18
Ht (%)	34.4 ± 7.2	36-54
WBC (10^3/mm^3)	3.63 ± 2.2	4-10
PLT (10^3/mm^3)	88.74 ± 56.24	150-400
pH	7.41 ± 0.05	7.37-7.44
pO_2 (mmHg)	91.4 ± 6.5	80-100
pCO_2 (mmHg)	34.9 ± 3.2	34-45
SaO_2 (%)	97.1 ± 0.95	96-98

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Gamma GT: Gamma glutamil transpeptidase; BUN: Blood urea nitrogen; Cr: Creatinine; PT: Prothrombin time; PTT: Partial thromboplastin time; Hb: Hemoglobin; Ht: Hematocrit; WBC: White blood cells; PLT: Platelets; pO_2: Arterial oxygen tension; $PaCO_2$: Arterial carbon dioxide tension; SaO_2: Arterial oxygen saturation; ALP: Alkaline phosphatase; TP: Total serum protein; ALB: Aalbumin; TBIL: Total serum bilirubin; IBIL: Indirect bilirubin.

Statistical analysis

Statistical analysis was accomplished by the paired *t* test, and $P < 0.01$ was considered as statistically significant, with a 99% confidence interval.

RESULTS

The results of the transthoracic Doppler echocardiography are shown in Table 2. No ventricular hypertrophy or segmental contraction abnormality was observed, and no patients presented valvular lesions or pericardial effusions. All patients presented normal systolic and diastolic ventricular function. In two patients, echocardiography revealed an estimated pulmonary artery pressure of 60 and 40 mmHg, respectively, accompanied by a discrete dilatation of the right ventricle. These two patients were subjected to EGDS.

Pre- and postoperative hemodynamic evaluation data are shown in Table 3 and Figure 1. Preoperative hemodynamic evaluation revealed an increased mean cardiac index (4.78 ± 1.13 L/min per m^2) and a reduction in the systemic vascular resistance index (1457 ± 380.7 dynes.s/cm^5.m^2). The systolic index (60.24 ± 12.8 mL/beats per m^2), left cardiac work index (6.14 ± 1.43 kg.m/m^2), left ventricle systolic work index (76.6 ± 17.8 g.m/m^2), right cardiac work index (1.22 ± 0.5 kg.m/m^2) and right ventricle systolic work index (15.25 ± 6.4 g.m/m^2)

Table 2 Preoperative transthoracic echocardiography in patients with portal hypertension due to hepatosplenic mansonic schistosomiasis

	Preop (mean ± SD)	Normal range
Ao	30.7 ± 1.9	20-35 mm
LA	38.1 ± 4.6	20-40 mm
LVDD	49.7 ± 3.9	35-55 mm
FDV	125.4 ± 29.6	50-150 mL
LVSD	30.9 ± 2.8	20-35 mm
SV	30.1 ± 8.1	50-150 mL
DD	37.6 ± 3.3	30%-40%
EF	75.9 ± 4.5	65%-80%
Se	8.9 ± 0.6	7-11 mm
Pw	8.4 ± 0.5	7-11 mm
V/M	65.1 ± 6.8	45%-75%

Ao: Aorta; LA: Left atrium; LVDD: Left ventricular diastolic diameter; FDV: Final diastolic volume; LVSD: Left ventricular systolic diameter; SV: Systolic volume; DD: Shortening fraction; EF: Ejection fraction; Se: Septum wall thickness; Pw: Left ventricular wall thickness; V/M: Left ventricular volume/mass relationship; E/A: Ratio between wave E and A.

Table 3 Pre- and postoperative hemodynamic parameters in patients with portal hypertension due to hepatosplenic mansonic schistosomiasis

	Preop	EGDS	DSRS	Normal values
HR	80.2 ± 11.4	83.1 ± 10.6	86.7 ± 17.9	80-100 beats/min
MABP	91.4 ± 12.5	93.6 ± 14.4	92.36 ± 13.75	80-100 mmHg
RAP	7.9 ± 2.5	7 ± 2.4	7.26 ± 2.4	0-7 mmHg
PCWP	10.2 ± 2.7	9.1 ± 3	9.55 ± 2	8-12 mmHg
PAP	18 ± 5.1	12.65 ± 4.7[d]	19.84 ± 5.2	12-15 mmHg
CI	4.78 ± 1.13	3.8 ± 0.4[d]	5.2 ± 0.76	2.5-4 L/min per m^2
SI	60.24 ± 12.8	46.2 ± 8.6[d]	59.64 ± 10.5	41-51 mL/beat per m^2
SVRI	1457 ± 380.7	1901.4 ± 330.2[d]	1389 ± 311	1970-2390 dynes.s/cm^5.m^2
PVRI	133 ± 62	141.65 ± 102.9	139.7 ± 67.8	225-315 dynes.s/cm^5.m^2
LCWI	6.14 ± 1.43	4.9 ± 0.7[b]	6.94 ± 1.3	3.4-4.2 kg.m/m^2
LVSWI	76.6 ± 17.8	59 ± 12.6[b]	82.65 ± 17	50-62 g.m/m^2
RCWI	1.22 ± 0.5	0.79 ± 0.4[b]	1.31 ± 0.35	0.54-0.60 kg.m/m^2
RVSWI	15.25 ± 6.4	9.45 ± 4.8[b]	16.3 ± 6.3	7.9-9.7 g.m/m^2

[b]P < 0.01 between EGDS and PREOP; [d]P < 0.001 between EGDS and PREOP. Values are expressed as means ± SD. EGDS: Esophagogastric devascularization and splenectomy; DSRS: Distal splenorenal shunt; HR: Heart rate; MABP: Mean arterial blood pressure; PAP: Mean pulmonary artery pressure; RAP: Mean right atrium pressure; PCWP: Pulmonary capillary wedged pressure; CI: Cardiac index; SI: Systolic index; SVRI: Systemic vascular resistance index; PVRI: Pulmonary vascular resistance index; RCWI and LCWI: Right and left cardiac work indexes, LVSWI and RVSWI: Left and right ventricular systolic work indexes.

were all increased. Heart rate (80.2 ± 11.4 beats/min), mean arterial blood pressure (91.4 ± 12.5 mmHg) and pulmonary capillary wedge pressure (10.2 ± 2.7 mmHg) were all within normal limits. Mean pulmonary artery pressure (18 ± 5.1 mmHg), as well as right atrial pressure (7.9 ± 2.5 mmHg), was increased, while the pulmonary vascular resistance index (133 ± 62 dynes.s/cm^5.m^2) was decreased.

Four days after EGDS, there was a significant decrease in cardiac index (3.8 ± 0.4 L/min per m^2) and a significant increase in systemic vascular resistance index (1901.4 ± 330.2 dynes.s/cm^5.m^2) toward normal levels. The systolic index (46.2 ± 8.6 mL/beats per m^2), left cardiac work index

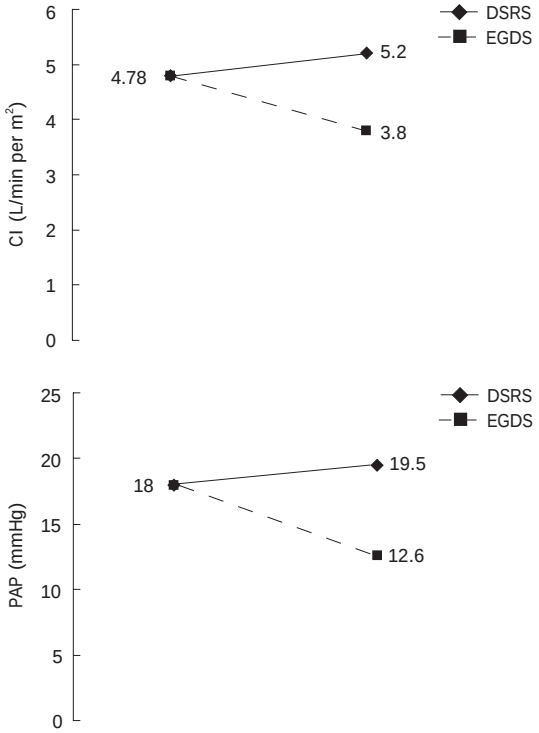

Figure 1 Schematic illustration showing important differences in cardiac index (CI) and pulmonary artery pressure (PAP) before and after EGDS (dashed line) and DSRS (continuous line).

(4.9 ± 0.7 kg.m/m^2) and left ventricle systolic work index (59 ± 12.6 g.m/m^2) also significantly decreased toward normal levels. There was no significant alteration in heart rate (83.1 ± 10.6 beats/min), mean arterial blood pressure (93.6 ± 14.4 mmHg), pulmonary capillary wedge pressure (9.1 ± 3 mmHg) and right atrial pressure (7 ± 2.4 mmHg). There was also a significant reduction in pulmonary artery pressure (12.65 ± 4.7 mmHg), right cardiac work index (0.79 ± 0.4 kg.m/m^2), right ventricle systolic work index (9.45 ± 4.8 g.m/m^2), and a non-significant increase in pulmonary vascular resistance index (141.65 ± 102.9 dynes.s/cm^5.m^2).

Four days after DSRS, there was a non-significant increase in cardiac index (5.2 ± 0.76 L/min per m^2), and a non-significant decrease in systemic vascular resistance index (1389 ± 311 dynes.s/cm^5.m^2). The systolic index (59.64 ± 10.5 mL/beats per m^2), left cardiac work index (6.94 ± 1.3 kg.m/m^2) and left ventricle systolic work index (82.65 ± 17 g.m/m^2) remained above normal levels. There were non-significant increases in heart rate (86.7 ± 17.9 beats/min) and mean arterial blood pressure (92.36 ± 13.75 mmHg), and non-significant decreases in pulmonary capillary wedge pressure (9.55 ± 2 mmHg) and right atrial pressure (7.26 ± 2.4 mmHg). In addition, there were non-significant increases in pulmonary artery pressure (19.84 ± 5.2 mmHg), right cardiac work index (1.31 ± 0.35 kg.m/m^2), and right ventricle systolic work index (16.3 ± 6.3 g.m/m^2), with no significant alteration in pulmonary vascular resistance index (139.7 ± 67.8 dynes.s/cm^5.m^2).

DISCUSSION

Hemodynamic changes after the surgical treatment of schistosomal portal hypertension with disconnection or

shunt procedures may contribute to the understanding of the hyperdynamic circulation observed in these patients with portal hypertension and preserved liver function. Twenty-nine patients (80.5%) showed an elevated cardiac index in the preoperative evaluation, which characterized a hyperdynamic circulatory state[8,9]. Although easily clinically recognized in patients with cirrhosis by peripheral arterial vasodilatation, hypotension and tachycardia, which are usually related to progressive liver failure, in schistosomal patients, these alterations are completely absent because they are less intense and liver function is preserved[10].

We have previously demonstrated that hepatosplenic schistosomiasis presents mild pulmonary hypertension and induces a hyperdynamic circulatory state, which is corrected after EGDS[2,7]. We have suggested that these changes are correlated with the portosystemic collateral circulation, especially as a consequence of splanchnic hyperflow[7]. Our group, as do Wattanasirichaigoon *et al*[8], believes that portosystemic collateral circulation mimics an arteriovenous fistula, in which the high-pressure portal blood connects with the lower pressure systemic venous circulation, which decompresses the portal circulation, but increases portal blood flow. As portal blood flow increases, so does collateral flow, and it is almost totally shunted with the systemic circulation. These observations should be considered particularly in patients with hepatosplenic schistosomiasis, in whom large splenomegaly induces splenic hyperflow, which plays an important role in the origin and maintenance of hyperdynamic circulation[3]. In the present study, 15 patients subjected to EGDS (88.2%) presented normalization of cardiac and systemic vascular resistance indexes, which suggested that EGDS corrected the hyperdynamic circulation. In cirrhosis, physical exercise and pharmacological stress determine an increase in left ventricular end diastolic pressure and a fall in cardiac stroke index and left ventricular ejection fraction, which indicates an abnormal ventricular response[9,11,12]. In these patients, the reduced vascular resistance may mask left ventricular failure. In contrast, the correction of hyperdynamic circulation without elevation of filling pressure, and the normal pre-operative echocardiographic parameters strongly suggest an absence of cardiomyopathy in patients with portal hypertension due to hepatosplenic mansonic schistosomiasis.

On the other hand, patients subjected to DSRS maintained a hyperdynamic circulation. These observations suggested that splenic flow through the venous shunt maintained a low-resistance circuit and hence, the hyperdynamic circulation. These findings are corroborated by the increases in heart rate, systolic, left cardiac work and left ventricle systolic work indexes observed in these patients. Studies that assess the hemodynamic pattern in a later period after EGDS or DSRS are necessary to confirm the findings of our study.

Portopulmonary hypertension (PPHTN) is defined as an increase in mean pulmonary artery pressure greater than 25 mmHg, increased pulmonary vascular resistance (greater than 120 dynas/cm^5), and pulmonary capillary wedge pressure lower than 15 mmHg, in the presence of portal hypertension[13-15]. There are several mechanisms proposed for its development, including an increased production of vasoconstrictors[16-18], increased pulmonary blood flow leading to vascular endothelial damage and remodeling[19], excess of pulmonary vascular volume[20], cirrhotic cardiomyopathy with myocardial thickening and diastolic dysfunction[21], and in situ microthrombosis[22]. Interestingly, to date there are no data showing any correlation between the extent of PPHTN and the intensity of portal pressure, severity of liver disease or degree of shunting[23].

Arterial blood gases and left (systolic and diastolic) ventricular function were within normal limits and, pulmonary capillary wedge pressure was < 15 mmHg in all patients during the hemodynamic study, which provided evidence of normal cardiopulmonary function.

In the present study, 23 patients (63.8%) presented with pulmonary artery pressure greater than 15 mmHg; 13 (36.1%) between 15 and 20 mmHg, eight (22.2%) between 20 and 25 mmHg. In two patients (5.5%), pulmonary artery pressure was > 25 mmHg, which demonstrated a tendency toward pulmonary hypertension in schistosomal portal hypertension, as previously described by our group[24].

After EGDS, we observed a significant reduction in pulmonary artery pressure in 88.2% of the patients. There was also a significant reduction in right cardiac work and right ventricular systolic work indexes, without significant increase in pulmonary vascular resistance index. The two patients without pulmonary artery pressure reduction showed a normal preoperative pulmonary artery pressure (< 15 mmHg). These findings suggest that pulmonary hyperflow may contribute to the elevated pulmonary artery pressure observed in these patients. The reduced pulmonary vascular resistance may be an accommodation of pulmonary vasculature to the hyperflow, in an attempt to maintain normal pulmonary artery pressure. Nevertheless, this adaptation seems ineffective, since pulmonary artery pressure was elevated in the majority of the patients studied. The two patients with PPHTN subjected to EGDS showed reduction of both cardiac index and mean pulmonary artery pressure.

These findings were confirmed by the hemodynamic pattern of patients subjected to DSRS, with which, 16 patients (84.2%) showed a slight increase in pulmonary artery pressure, right cardiac work and right ventricular systolic work indexes. In fact, we have previously reported the cases of two young asymptomatic patients with normal cardiovascular preoperative assessment (electrocardiography, thoracic X-ray and transthoracic echocardiography) who died 4 and 7 d after DSRS, and necropsy showed signs of acute pulmonary hypertension and right ventricular failure[25]. We hypothesized that these patients had undiagnosed preoperative elevated mean pulmonary artery pressure that caused acute pulmonary hypertension after the splenorenal shunt, due to pulmonary hyperflow. The importance of preoperative hemodynamic evaluation in hepatosplenic schistosomiasis is to identify patients with raised pulmonary artery pressure, and consequently, to choose EGDS rather than DSRS as the ideal surgical treatment.

In conclusion, the hyperdynamic circulatory state present in hepatosplenic mansonic schistosomiasis was corrected by EGDS, but it was maintained by DSRS.

Similarly, the raised mean pulmonary artery pressure was corrected by EGDS and maintained by DSRS. EGDS seems to be the most physiologic surgical alternative for patients with schistosomal portal hypertension, because of its tendency to lead to immediate normalization of the systemic and pulmonary hemodynamic parameters. Cardiomyopathy present in cirrhotic portal hypertension seems to be absent in hepatosplenic mansonic schistosomiasis. Hemodynamic studies to evaluate the late circulatory pattern in these patients are necessary to confirm our findings.

COMMENTS

Background

To the best of our knowledge, there are no data in the literature regarding postoperative systemic hemodynamics after surgical treatment of schistosomal portal hypertension. The purpose of this study was to prospectively investigate the postoperative systemic hemodynamic effects in two different surgical procedures largely employed for treatment of schistosomal portal hypertension.

Research frontiers/Innovations and breakthroughs

Hemodynamic changes after the surgical treatment of schistosomal portal hypertension with disconnection or shunt procedures may contribute to the understanding of the hyperdynamic circulation observed in these patients with portal hypertension and preserved liver function. This knowledge may be useful in deciding on the best surgical option for each patient.

Applications

The importance of preoperative hemodynamic evaluation in hepatosplenic schistosomiasis is to identify patients with raised pulmonary artery pressure, and consequently, choose EGDS rather than DSRS as the ideal surgical treatment. EGDS seems to be the most physiologic surgical alternative for patients with schistosomal portal hypertension, due to its tendency to lead to immediate normalization of the systemic and pulmonary hemodynamic parameters.

Terminology

Schistosomal portal hypertension: presinusoidal portal hypertension in patients with preserved liver function.

Peer review

This study examines in patients with pre-hepatic portal hypertension, caused by schistosomiasis, the short-term effects of two different interventions, EGDS and DSRS, on hemodynamic cardiac parameters derived from transthoracic echocardiography and direct measurement of pulmonary artery pressure. The study shows that EGDS lowers cardiac output and mean pulmonary artery pressure (probably as a result of splenectomy), while DSRS has no effect on these parameters.

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RAPID COMMUNICATION

Neural cell adhesion molecule-180 expression as a prognostic criterion in colorectal carcinoma: Feasible or not?

Oge Tascilar, Güldeniz Karadeniz Cakmak, Ishak Ozel Tekin, Ali Ugur Emre, Bulent Hamdi Ucan, Oktay Irkorucu, Kemal Karakaya, Mesut Gül, Hüseyin Bülent Engin, Mustafa Comert

Oge Tascilar, Güldeniz Karadeniz Cakmak, Ali Ugur Emre, Bulent Hamdi Ucan, Oktay Irkorucu, Kemal Karakaya, Mesut Gül, Mustafa Comert, Department of Surgery, Zonguldak Karaelmas University, The School of Medicine, Kozlu-Zonguldak 67600, Turkey
Ishak Ozel Tekin, Department of Immunology, Zonguldak Karaelmas University, The School of Medicine, Kozlu-Zonguldak 67600, Turkey
Hüseyin Bülent Engin, Department of Medical Oncology, Zonguldak Karaelmas University, The School of Medicine, Kozlu-Zonguldak 67600, Turkey
Correspondence to: Dr. Güldeniz Karadeniz Cakmak, Zonguldak Karaelmas Universitesi, Arastirma ve Uygulama Hastanesi Bashekimligi, Kozlu-Zonguldak 67600, Turkey. gkkaradeniz@yahoo.com
Telephone: +90-372-2610169 Fax: +90-372-2610155
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Abstract

AIM: To evaluate the frequency of neural cell adhesion molecule (NCAM)-180 expression in fresh tumor tissue samples and to discuss the prognostic value of NCAM-180 in routine clinical practice.

METHODS: Twenty-six patients (16 men, 10 women) with colorectal cancer were included in the study. Fresh tumor tissue samples and macroscopically healthy proximal margins of each specimen were subjected to flow-cytometric analysis for NCAM-180 expression.

RESULTS: Flow-cytometric analysis determined NCAM-180 expression in whole tissue samples of macroscopically healthy colorectal tissues. However, NCAM-180 expression was positive in only one case (3.84%) with well-differentiated Stage Ⅱ disease who experienced no active disease at 30 mon follow-up.

CONCLUSION: As a consequence of the limited number of cases in our series, it might not be possible to make a generalisation, nevertheless the routine use of NCAM-180 expression as a prognostic marker for colorectal carcinoma seems to be unfeasible and not cost-effective in clinical practice due to its very low incidence.

Key words: Neural cell adhesion molecule-180; Colorectal cancer; Prognosis; Flow-cytometry

INTRODUCTION

Cancer is currently one of the major causes of morbidity and mortality in humans. Tumor progression to local invasion and metastasis are clinically the most relevant processes for prognosis. However the molecular pathways involved in tumor progression are the least well defined at the cellular level, which represents one of prime challenges in cancer research. Tumor suppressor genes are the major target for treatment modalities in most malignant diseases, including gastrointestinal neoplasies. For colon carcinoma, Deleted in Colon Carcinoma (DCC) accounts for one of the best described tumor suppressors involved in adhesive interactions. DCC is a member of the immunglobulin (Ig) superfamily. The neural cell adhesion molecule (NCAM, CD56) is another member of this family possessing structural and sequence homology to DCC[1,2]. Members of the Ig family of cell adhesion molecules (CAMs) play an important role in progression to tumour malignancy and metastasis. NCAM is an embryologic adhesion molecule and a cell membrane protein that modulates neuroendocrine cell growth, migration, and differentiation[3]. NCAM mediates cell-cell and cell-matrix adhesion, contact inhibition and tissue morphogenesis and also is proposed to be critical in signal transduction[3,4]. The major variants of NCAM are classified based on the sialic acid content as either NCAM-H (high-sialic-acid content) or NCAM-L (low-sialic-acid content). The properties of NCAM-H molecules are the following: relative molecular weight between 200-250 kDa, more prevelant in embrionic tissue, blocks adhesion-binding sites and fascilitates cell migration during embriogenesis[5-7]. Therefore, cell-cell or cell-matrix adhesions can be altered by downregulation of NCAM molecules or by upregulation of sialic acid content within the NCAM protein. NCAM-L with a molecular weight of 120-180 kDa predominates in adult tissue and is expressed in three major isoforms, resulting from alternative mRNA splicing and depending on cell type and stage of differentiation[5,8,9]. The major

isoforms have the 5-distal immunoglobulin and 2-membrane proximal fibronectin (FN)-Ⅲ domains. NCAM-120 is glycophosphatidylinositol-linked to the plasma membrane by a sequence encoded by exon 15[9]. NCAM-140 has the basic NCAM-120 structure with a transmembrane sequence and a short (40-kDa) intracellular tail. NCAM-180 has a longer intracellular tail (90 kDa) encoded by exons 17, 19, and unique to this isoform, exon 18. The intracellular component of NCAM-180 anchors the molecule to the cytoskeleton. NCAM-180 is believed to be an important structural molecule that mediates cell-cell adhesion by providing a mechanical linkage between the cytoskeleton and the extracellular adhesive end of the molecule resulting in tissue stabilisation[10]. NCAM-180 was found to be expressed in normal colonic epithelium villous tips and the expression was demonstrated to be lost in highly aggressive colon cancers[7,11]. This study was undertaken to further evaluate the frequency of NCAM-180 expression in fresh tumor tissue samples by flow-cytometric analysis and to discuss the prognostic value of NCAM-180 in colorectal carcinoma in routine clinical practice.

MATERIALS AND METHODS

Patients and tumor samples

Fresh tumor tissue samples were obtained at operation from 26 patients with colorectal cancer who underwent surgery between January 2002 and January 2006. Two samples from each case, one of which was chosen directly from the center of a main tumor lesion and the other from the macroscopically healthy proximal margins, were transferred to flow-cytometric analysis immediately. The remaining specimen was fixed in 10% phosphate buffered formaldehyde, and embedded in paraffin for histopathological analysis. Patient characteristics are shown in Table 1. Oncologic follow-up was performed in each case within 6-12 mo periods. Clinical data were obtained by direct interviews with patients as a part of oncologic follow-up. Patients were defined as having an aggressive clinical course if they presented with an obstructing or perforating lesion or had metastatic disease. Death within 18 mo of presentation was also classified as having an aggressive clinical course. Participation in the study was voluntary and all patients gave their informed consent to participate. The study was approved by the Local Ethics Committee of Zonguldak Karaelmas University Hospital, Zonguldak, Turkey.

Table 1 Background of 26 cases of resected colorectal carcinoma

Case	Gender	Age (yr)	Location	Tumour
1	F	49	Colon	Adenocarcinoma
2	F	70	Rectum	Adenocarcinoma
3	M	86	Colon	Adenocarcinoma
4	M	76	Colon	Adenocarcinoma
5	M	73	Colon	Adenocarcinoma
6	F	76	Colon	Adenocarcinoma
7	M	72	Colon	Adenocarcinoma
8	F	68	Colon	Adenocarcinoma
9	M	72	Colon	Adenocarcinoma
10	M	50	Rectum	Adenocarcinoma
11	M	88	Colon	Adenocarcinoma
12	F	68	Rectum	Adenocarcinoma
13	F	48	Colon	Adenocarcinoma
14	F	75	Rectum	Adenocarcinoma
15	M	37	Rectum	Adenocarcinoma
16	M	57	Rectum	Adenocarcinoma
17	F	71	Colon	Adenocarcinoma
18	M	70	Colon	Adenocarcinoma
19	M	47	Colon	Adenocarcinoma
20	M	47	Colon	Adenocarcinoma
21	F	71	Colon	Adenocarcinoma
22	M	53	Colon	Adenocarcinoma
23	M	80	Rectum	Adenocarcinoma
24	F	49	Rectum	Adenocarcinoma
25	M	76	Colon	Adenocarcinoma
26	M	62	Rectum	Adenocarcinoma

Flow-cytometric analysis

All biopsy materials were dissociated mechanically with Medimachine (Becton Dickinson, CA, USA). The dissociated cells were prepared as single cell suspension in PBS (phosphate buffered salt solution) .The cell number was calibrated as 10×10^6/mL. Each 100 μL sample incubated with 10 μL anti-CD56-PE (phycoerythrin conjugated NCAM monoclonal antibody) for 15 min at room temperature. Samples were processed by a Coulter Q Prep Workstation and run with a Beckman-Coulter Epics XL MCL flow cytometer (Beckman coulter, Florida, USA). At least 20000 events were acquired for each sample. Data analysis was performed using EXPO32 (Beckman-Coulter) software. Only CD45 negative population gated were used for NCAM analysis. The upper limit of background fluorescence was set such that no more than 1% of the events with the matched isotype was in the positive region.

Histological classification

Pathologic stagings were performed based on the TNM staging system developed by the American Joint Committee on Cancer[12]. Histologic tumor typing was applied according to the classification system indicating poor, moderate or well differentiation. Macroscopically healthy proximal margins were verified to be tumor free by histopathologic examination.

RESULTS

Of the 26 patients, 16 (61.5%) were male and 10 (38.5%) were female. The mean age was 65.04 ± 13.60 (range, 37-88) years. Tumors were found to be localized in colonic segments in 19 (73.07%) and in rectum in the rest 7 (26.93%) cases. Four patients died because of cardiovascular or pulmonary complications following surgery. No patients died during follow-up. The mean follow-up period was 19.05 ± 12.33 (range, 4-56) mo. Histopathologic stage, differentiation status, NCAM-180 expression and postoperative survival periods are shown in Table 2. The number of patients in Stage Ⅰ, Ⅱ, Ⅲ and Ⅳ disease were 3 (11.53%), 7 (26.92%), 9 (34.61%), and 7 (26.92%), respectively. Tumors were detected to be well-differentiated in 4 (15.38%), moderately-differentiated in 15 (57.69%) and poorly-differentiated in 7 (26.92%) cases. Flow-cytometric analysis determined NCAM-180 expression in whole tissue samples of macroscopically

Table 2 Results of histopathologic evaluation and flow cytometric analysis of NCAM-180 status

Case	pTNM	Stage	Histology	NCAM-180	Outcome
1	T3N0M0	Ⅱ	Moderate	-	No active disease at 21 mo follow-up
2	T4N1M1	Ⅳ	Poor	-	Died of metastatic diesease 8 mo postresection
3	T4N2M1	Ⅳ	Moderate	-	No active disease at 9 mo follow-up
4	T3N0M0	Ⅱ	Well	-	No active disease at 20 mo follow-up
5	T4NIM1	Ⅳ	Poor	-	Died of cardiopulmonary complication postoperatively
6	T3N1M0	Ⅲ	Moderate	-	No active disease at 6 mo follow-up
7	T2N0M0	Ⅰ	Moderate	-	Metachrone colonic disease at 15 mo
8	T2N0M0	Ⅰ	Moderate	-	No active disease at 15 mo follow-up
9	T4N2M0	Ⅲ	Moderate	-	Died of metastatic diesease 18 mo postresection
10	T3N2M0	Ⅲ	Moderate	-	No active disease at 18 mo follow-up
11	T2N0M0	Ⅰ	Moderate	-	Died of cardiopulmonary complication postoperatively
12	T3N0M1	Ⅳ	Poor	-	Died of cardiopulmonary complication postoperatively
13	T3N1M0	Ⅲ	Moderate	-	No active disease at 32 mo follow-up
14	T3N1M0	Ⅲ	Moderate	-	No active disease at 20 mo follow-up
15	T3N0M0	Ⅱ	Poor	-	No active disease at 16 mo follow-up
16	T3N1M0	Ⅲ	Well	-	No active disease at 19 mo follow-up
17	T3N0M0	Ⅱ	Well	+	No active disease at 30 mofollow-up
18	T3N1M0	Ⅲ	Well	-	No active disease at 44 mo follow-up
19	T4N2M1	Ⅳ	Poor	-	Died of metastatic diesease 5 mo postresection
20	T4N2M1	Ⅳ	Poor	-	Died of metastatic diesease 4 mo postresection
21	T3N0M0	Ⅱ	Moderate	-	Died of metastatic diesease 56 mo postresection
22	T4N1M1	Ⅳ	Moderate	-	Died of metastatic diesease 15 mo postresection
23	T4N1M0	Ⅲ	Moderate	-	Died of cardiopulmonary complication postoperatively
24	T3N0M0	Ⅱ	Poor	-	No active disease at 18 mo follow-up
25	T3N0M0	Ⅱ	Moderate	-	No active disease at 16 mo follow-up
26	T4N1M0	Ⅲ	Moderate	-	No active disease at 14 mo follow-up

healthy colorectal tissues. However, NCAM-180 expression was positive in only one case (3.84%) with well-differentiated Stage Ⅱ disease, and this patient experienced no active disease at 30 mo follow-up.

Correlation between NCAM-180 expression in colorectal cancer and other parameters

It is not possible to compare overall survival outcomes in this series with only one (3.84%) positive NCAM-180 expression. However, NCAM-180 expression was positive in a well-differentiated Stage Ⅱ tumor with an uneventfull clinical course for 30 mo following surgery. Considering well-differentiated tumors, one of three patients without NCAM-180 expression experienced a longer disease free survival period (44 *vs* 30 mo). Moreover, NCAM-180 epxression was not detected in both moderate or poor differentiated tumors. Evaluation of the patients with stage Ⅱ disease demonstrated that one of six patients without NCAM-180 expression survived 56 mo after diagnosis and no active disease was detected in the other 5 patients within a mean follow-up period of 18.2 (range, 16-21) mo.

DISCUSSION

Tumoral invasion and metastasis are the most critical and complex processes in aggressive human cancers and are one of the major causes of cancer deaths. Cell adhesion molecules, including the immunoglobulin superfamily, play a crucial role in determining tumor development and the metastatic cascade[13,14]. Variations in cell-cell and cell-matrix adhesion accompany the progression from benign tumours to invasive, malignant cancer and the subsequent metastatic dissemination of tumour cells. The hallmark of neoplastic and metastatic growth is thought to be reduced adhesiveness between cells and also between cells and the extracellular matri[3]. Several groups of adhesion molecules are importantly involved in regulation of tumor invasion and metastasis.

NCAM (CD56) is a calcium independent cell adhesion molecule, which mediates homotypic and heterotypic cell-cell and cell-matrix adhesion[15-17]. NCAM has been found to be a significant factor for survival in various solid tumors. A correlation between reduced NCAM expression and poor prognosis has been reported for some cancer types[11,18,19]. The existence of NCAM-180 has been proposed to be a good prognostic criterion in colorectal carcinoma[11]. Previous studies have demonstrated that NCAM-180 is present in normal colonic epithelium and in benign colonic tumors and loss of NCAM-180 expression might result in defective intracellular adhesion between colonocytes in aggressive colon carcinoma[7,11]. In this study we investigated the NCAM-180 expression rate in fresh tumor tissue samples of colorectal carcinoma and the association of an aggresive clinical course with loss of this expression.

NCAM expression has been investigated in various solid and neuroendocrine tumours. There is a consensus that presence of its polisialiated (embryonic) form, which is less adhesive than the adult form [that contains a relatively low polisialic acid (PSA) content], is associated with a poor prognosis. Correlation between N-CAM expression and perineural spread has been confirmed in a variety of human carcinomas. The existence of the polisialiated form of NCAM in Wilms' tumor, neuroblastoma, pituitary tumor, small cell lung cancer, gallbladder and bile duct cancer,

squamous cell cancer of head and neck, and prostat cancer results in perineural invasion and agrressive metastatic behaviour with a poor clinical outcome[20-28]. As the expression of the polisialiated form of NCAM correlates with tumor growth and invasiveness because of its role in cell disassociation, ıt was considered to be a poor prognostic criterion in pituitary tumors and rhabdomyosarcoma[22,29]. Polysialation has been proposed to involve steric inhibition of membrane-membrane apposition and cell adhesiveness, based on the biophysical properties of the polisialic acid[30]. In renal cell carcinoma, NCAM expression was suggested to be a risk factor for tumor metastasis[31]. Moreover, NCAM is not polysialylated in renal cell carcinoma suggesting that it plays another role in these tumors involving homophilic adhesion[31]. Conversely, for other tumors like pancreatic adenocarcinomas, reduced levels of NCAM expression were found to correlate with increased tumor malignancy[19]. This result was also observed in a transgenic mouse model of β-cell pancreatic carcinoma by crossing these mice with NCAM knockout mice[32]. The hypothesis was reduced levels of NCAM could increase cell dissociation from primary tumors. Moreover, an overall decrease in the NCAM level has been observed in another subset of tumors including colon carcinoma and astrocytoma. In these tumors NCAM expression is markedly down-regulated, and the loss of NCAM correlates with poor prognosis[7,11,18,33]. In gastrointestinal neoplasia, when pancreatic, colorectal and gastric cancer were considered, poorly differentiated tumors had lower levels of NCAM than well or moderately differentiated tumors[18].

Previous studies have demonstrated that NCAM-180 is present in normal colonic epithelium and NCAM-180 expression was found to be absent in clinically aggressive colon carcinomas[11]. Consistent with this thesis, colorectal carcinomas expressing NCAM-180 should experience a good clinical course with longer disease free survival. In other words, overexpression of the polysialylated form of NCAM or reduced expression of NCAM-180 has been suggested to decline intracellular adhesion, facilitating metastatic behavior in cancer. This study was designed to determine the rate of NCAM-180 expression in fresh colorectal tumour tissue and correlation of NCAM-180 expression with clinical course. In our series of 26 colorectal carcinoma, we determined NCAM-180 expression in only one patient (3.84%) (pathologic stage Ⅱ-well differentiated tumour) with a good clinical course during a follow-up period of 30 mo. This was an expected finding according to the previous literature[7,11]. However, we detected that 6 of the other patients with the same clinical and pathological stage at diagnosis and surgery, experienced either similar or a better clinical course during follow-up as well. Moreover, 2 patients without NCAM-180 expression and in an advanced pathological stage at diagnosis survived more than the patient with NCAM-180 expression. These are controversial results predicting that attribution of NCAM-180 expression as a good prognostic criterion in colorectal carcinoma is something to be interrogated before acceptance.

NCAM-180 has been proposed as a candidate tumor supressor in colorectal carcinoma previously and might play a crucial role in tumor behaviour by mediating colonic epithelial integrity and preventing tumour invasiveness and metastasis due to cellular adhesive properties. When colorectal cancer is considered, loss of NCAM-180 expression might lead to reduced homotypic binding between cancerous cells, resulting in detachment from the primary cancerous mass and invading other organs, acting systematically. However, in our series the NCAM-180 expression rate was only 3.84% and statistical correlation analysis of survival with NCAM-180 expression was not possible according to this low frequency. Moreover, the comparision according to tumor differentiation and stage revealed that loss of NCAM-180 expression in either well-differentiated or stage Ⅱ disease did not result in a worst clinical course. As a consequence of the limited number of cases in our series, it might not be possible to make a generalisation, nevertheless the routine use of NCAM-180 expression as a prognostic marker for colorectal carcinoma seems not to be feasible and cost-effective in clinical practice due to being present at a very low frequency. Further studies with a greater number of cases are thus called for to study the underlying mechanisms of tumor metastasis and prognosis in colorectal carcinoma.

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COMMENTS

Background

Cancer being one of the most mortal disease worldwide, tumor markers and prognostic criterions attract a great enthusiasm above researchers. Tumor suppressor genes and cell adhesion molecules are considered to play a crucial role in tumor pathophysiology.

Research frontiers

Neural cell adhesion molecule (NCAM-CD56) mediates cell-cell and cell-matrix adhesion, contact inhibition and tissue morphogenesis and also proposed to be critical in signal transduction. The major variants of NCAM are classified based on the sialic acid content as either NCAM-H (high-sialic-acid content) or NCAM-L (low-sialic-acid content). NCAM-L with a molecular weight of 120-180 kDa, predominates in adult tissue and is expressed in three major isoforms. NCAM-180 is believed to be an important structural molecule that mediates cell-cell adhesion by providing a mechanical linkage between the cytoskeleton and the extracellular adhesive end of the molecule resulting in tissue stabilisation.

Innovations and breakthroughs

A correlation between reduced NCAM expression and poor prognosis has been reported for some cancer types. NCAM-180 expression has been demonstrated to be lost in highly aggressive colon cancer and proposed to function as a tumor supressor. From this point of view we aim to evaluate the frequency of NCAM-180 expression in fresh tumor tissue samples by flow-cytometric analysis and to discuss the prognostic value of NCAM-180 in colorectal carcinoma in routine clinical practice.

Applications

The most critical deficit in the ability to treat cancer effectively is the lack of knowledge about cellular basis and markers for early diagnosis. The verification of an association between various types of malignancies and adhesion molecules might provide novel targets to cancer therapy by indicating the accurate goals.

Terminology

Neural cell adhesion molecule (NCAM-CD56) is a well identified cell membrane protein and a member of immunoglobulin superfamily, possessing structural and sequence resemblance to Deleted in Colon Carcinoma (DCC), which is another member of the same superfamily.

Peer review

The authors evaluated the frequency of NCAM-180 expression in fresh tumor tissue samples by flow-cytometric analysis and found that NCAM-180 expression in whole tissue samples of macroscopically healthy colorectal tissues, but only in one case (3.84%) with well-differentiated Stage Ⅱ disease. As discussed by the authors that the limited number of cases in the series, it is impossible to make a generalization. Further study with a large series of cases should be carried out to evaluated the clinicopathological significance of NCAM-180 expression in colorectal cancers.

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RAPID COMMUNICATION

Clinical significance of activity of ALT enzyme in patients with hepatitis C virus

Onder Akkaya, Murat Kiyici, Yusuf Yilmaz, Engin Ulukaya, Omer Yerci

Onder Akkaya, Yusuf Yilmaz, Department of Internal Medicine, Uludag University Medical School, Bursa, Turkey
Murat Kiyici, Department of Gastroenterology, Uludag University Medical School, Bursa, Turkey
Engin Ulukaya, Department of Biochemistry, Uludag University Medical School, Bursa, Turkey
Omer Yerci, Department of Pathology, Uludag University Medical School, Bursa, Turkey
Correspondence to: Yusuf Yilmaz, MD, Uludag Universitesi Tip Fakultesi Ic Hastaliklari Gorukle, Bursa, 16059 Turkey. yusufyilmaz@uludag.edu.tr
Telephone: +90-533-4403995 Fax: +90-224-4534028
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Abstract

AIM: To investigate serum alanine aminotransferase (ALT) levels in relation to the clinical, biochemical, ultrasonographic and histological characteristics of patients with hepatitis C virus.

METHODS: Duration of disease, HCV-RNA, liver steatosis, and the hepatitis activity index (HAI) were correlated with serum ALT in 36 patients with HCV. ALT values were also investigated in 16 control subjects without any liver diseases.

RESULTS: In bivariate analyses, ALT levels correlated with duration of HCV infection ($P < 0.01$), HCV-RNA ($P < 0.05$), and the HAI ($P < 0.01$). Among the components of the HAI, ALT concentrations were significantly associated with periportal bridging/necrosis ($P < 0.01$) and fibrosis ($P < 0.05$). In multivariate analysis, periportal bridging/necrosis ($\beta = 0.508$; $P < 0.01$), duration of HCV infection ($\beta = 0.413$; $P < 0.01$), and HCV-RNA ($\beta = 0.253$; $P < 0.05$) were independently associated with ALT activity. The normal ALT activity for men and women was < 23 IU/L and < 22 IU/L, respectively.

CONCLUSION: In patients with HCV, alterations in the liver tissue as reflected by ALT elevation are mainly associated with periportal bridging/necrosis, viral load and duration of disease. A cut-off value < 23 IU/L distinguished with high diagnostic accuracy healthy controls from patients with HCV.

Key words: Hepatitis C virus; Disease duration; Viral Load; Inflammation; Normal alanine aminotransferase

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease, frequently progressing to cirrhosis and increased risk of hepatocellular carcinoma[1-3]. Chronic hepatitis C is often silent, most of the times discovered only by routine serologic or biochemical testing[4-6]. Many attempts to identify the natural history and progression of hepatitis C infection have been made, but several aspects remain to be elucidated[7]. In individuals with chronic hepatitis C, viral load and elevated serum alanine aminotransferase (ALT) levels may have clinical relevance[8-10]. When parenchymal liver cells are damaged, aminotransferases leak from the liver into the blood, resulting in elevated levels of these enzymes in the bloodstream. The exact definition of the normal levels of serum ALT activity is crucial for screening and follow-up studies in hepatitis C infection[11,12]. It should be noted, however, that half of the untreated patients with chronic HCV infections display normal or minimally elevated serum ALT levels[13,14]. Accordingly, several studies have recently questioned whether previously established values to define normal ALT range are clinically accurate[11,12]. In this regard, it has been posited that the limits of normal for serum ALT should be revised accordingly[12].

In the present study, we sought to investigate serum ALT levels in relation to the clinical, biochemical, ultrasonographic and histological characteristics of patients with hepatitis C. We also aimed to study the normal level of ALT in Turkish healthy adults at low risk for chronic liver diseases. This information, in addition to daily clinical practice, may be clinically useful for research studies of hepatitis C and chronic liver diseases in Turkey.

MATERIALS AND METHODS

Study sample

A total of 36 patients (24 females, 12 males; mean age: 47.9 ± 13.2 years) with HCV infection were studied before the treatment with antiviral drugs. Patients with hemochromatosis, Wilson's disease, autoimmune hepatitis,

Table 1 General characteristics of the study patients with HCV infection

Characteristic	Entire cohort (n = 36)
Female, *n* (%)	24 (66.6%)
Age (yr)	47.9 ± 13.2
Estimated duration of HCV infection (mo)	58 (24-120)
HCV RNA (IU/mL)	2471075 ± 2490186
Body mass index (kg/m^2)	28.5 ± 3.9
Waist Circumference (cm)	98 ± 11
Fasting glucose (mg/dL)	96 ± 10
Haemoglobin (mg/dL)	13.9 ± 1.5
HOMA index	3.1 ± 2.4
Total cholesterol (mg/dL)	161 ± 36
HDL cholesterol (mg/dL)	48 ± 11
Triglycerides (mg/dL)	98 ± 41
Serum albumin (g/dL)	4.4 ± 0.3
Serum globulin (g/dL)	3.1 ± 0.6
Serum creatinine (mg/dL)	0.8 ± 0.2
AST (IU/L)	48 (32-67)
ALT (IU/L)	64 (34-80)
ALP (IU/L)	90 ± 33
GGT (IU/L)	45 (29-63)
LDH (IU/L)	184 ± 32
Total Bilirubin (mg/dL)	0.7 ± 0.3
Direct Bilirubin (mg/dL)	0.3 (0.2-0.4)
Platelet count (10^3/mm^3)	202 ± 54
Ferritin (ng/mL)	45 (34-77)
Positive ANA, *n* (%)	2 (5.5%)
Positive AMA, *n*	0
Positive ASMA, *n*	0
Positive LKM-1, *n*	0

Data expressed as means ± SD, or median (interquartile range), as appropriate; HOMA: Homeostasis model assessment; HDL: High-density lipoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma glutam.yl transpeptidase; LDH: Lactate dehydrogenase; ANA: Antinuclear antibodies; AMA: Antimitochondrial antibodies; ASMA: Anti-smooth muscle antibody; LKM-1: Liver-kidney microsomal antigen.

primary biliary cirrhosis, sclerosing cholangitis, biliary obstruction, alpha-1 antitrypsin deficiency, or malignancies were excluded from the present study. None of the subjects was using any medications, including estrogens, amiodarone, steroids, tamoxifen, or herbal supplements. Furthermore we excluded patients with daily alcohol intake exceeding 20 g/d. For control purposes, 16 healthy age- and gender-matched volunteers (9 females, 7 males) were recruited. All controls were judged to be in good health and confirmed as having normal liver by ultrasound. Subjects with a consumption of alcohol > 20 g/d or who were taking any medication were not included in the control group. All subjects underwent physical examination, anthropometric measurements and biochemical screening.

A written informed consent was obtained from all participants. Our study was in accordance with the ethical standards for human experimentation and approved by the Ethics Committee of the Uludag University Medical School.

Laboratory and virology assessment

Blood samples for the evaluation of alanine aminotransferase (ALT), and biochemical parameters were obtained after overnight fasting. Routine biochemical tests were carried out using commercially available kits (Hitachi, Tokyo, Japan). The HCV-RNA was determined in the sera using an RT-PCR assay (Amplicor, Roche, Mannheim), with a sensitivity of 70 copies/mL.

Ultrasound assessment

Liver ultrasound (US) scanning was performed to assess the degree of steatosis. All US procedures were performed by the same operator. Liver steatosis was assessed semi-quantitatively on a scale of 0 to 3:0, absent; 1, mild; 2, moderate and 3, severe.

Histological analysis

Ultrasonography-guided liver biopsies were performed under conscious sedation using a 16-gauge Klatskin needle. The length of histological specimens was not smaller than 2.5 cm. All biopsy specimens were placed in formalin solution for fixation and embedded in paraffin blocks. Serial sections (sectioned at 4 μm intervals) were stained with hematoxylin-eosin and Masson's trichrome. The hepatitis activity index (HAI), designed by Knodell and Desmet[15,16], was used to grade the severity of the necroinflammatory process and fibrosis. HAI comprises four separate scores, including periportal necrosis with or without bridging necrosis (0-10), intralobular degeneration and focal necrosis (0-4), portal inflammation (0-4) and fibrosis (0-4).

Statistical analysis

Variables are presented as counts and percentages, mean ± SD. Correlations among the study variables were assessed by means of the Pearson's correlation coefficients. Multivariate stepwise regression models were used to assess the independent predictors of ALT levels in patients with HCV infection. Cut-off values for serum ALT values were determined by means of the ROC curve analysis with the use of the MedCalc statistical software (Mariakerke, Belgium). A $P < 0.05$ was considered statistically significant. All computations were made using SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Bivariate analysis of serum ALT levels in patients with HCV infection

The characteristics of individuals with HCV infection are given in Table 1. The estimated median duration of HCV infection was 58 (interquartile range: 24-120) mo. Steatosis was present in 22 (61%) of the 36 HCV infected patients, of whom 8 (22%) had grade 1, 11 (30%) grade 2, and 3 (9%) grade 3. The histological findings of the study participants are shown in Table 2. In bivariate correlation analyses, ALT levels correlated with duration of HCV infection (r = 0.46, $P < 0.01$, Figure 1), HCV-RNA (r = -0.33, $P < 0.05$, Figure 2), and the HAI (r = 0.44, $P < 0.01$, Figure 3). Among the components of the HAI, ALT concentrations were significantly associated with periportal bridging/necrosis (r = 0.50, $P < 0.01$) and fibrosis (r = 0.37, $P < 0.05$).

Multivariate analysis

Multivariate stepwise regression analysis was used to

Table 2 Liver histology in the 36 study participants with HCV infection

Variable	Score
Hepatitis activity index	9.5 ± 3.7
Periportal necrosis with or without bridging necrosis	3.7 ± 2.2
Intralobular degeneration and focal necrosis	2.7 ± 1.1
Portal inflammation	3.0 ± 1.0
Fibrosis	1.9 ± 1.2

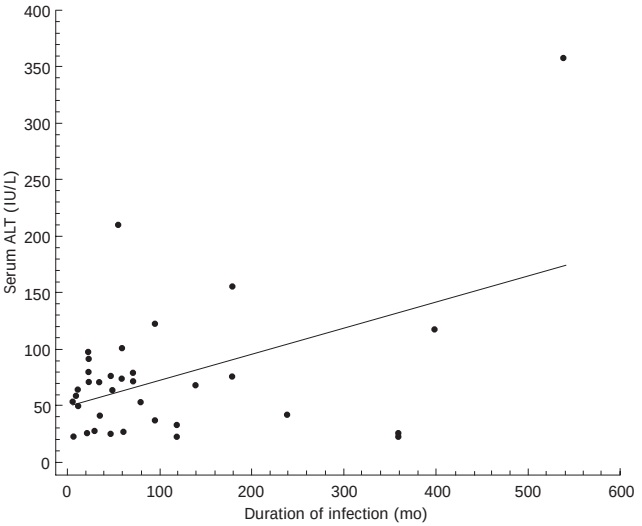

Figure 1 Scatter diagram and regression line showing a significant positive relationship between duration of HCV infection and serum alanine aminotransferase (r = 0.46, P < 0.01).

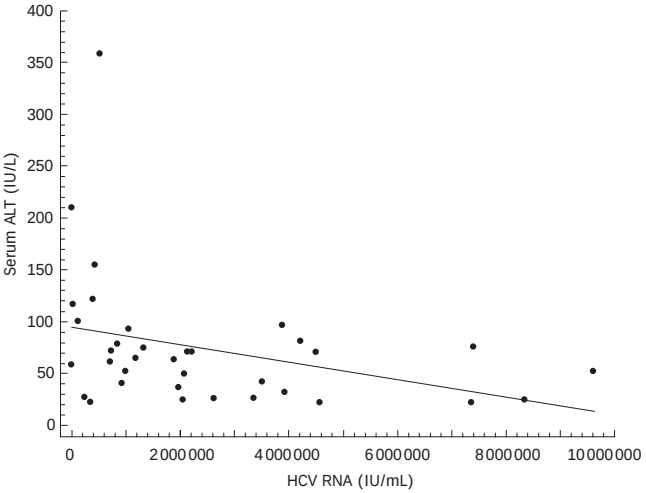

Figure 2 Scatter diagram and regression line showing a significant inverse relationship between viral load and serum alanine aminotransferase (r = -0.33, P < 0.05).

identify independent predictors of ALT levels in our patients with HCV infection. Serum ALT activity was considered as the dependent variable. All variables listed in Table 1 were entered into the multivariate model as independent variables. The results of this analysis showed that periportal bridging/necrosis (β = 0.508; P < 0.01), duration of HCV infection (β = 0.413; P < 0.01), and

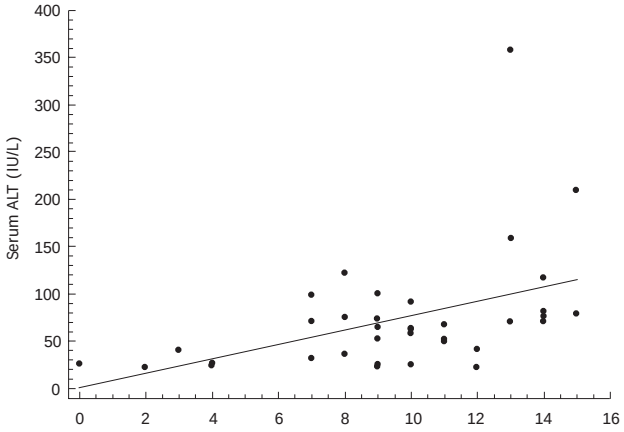

Figure 3 Scatter diagram and regression line showing a significant positive relationship between the Histology Activity Index and serum alanine aminotransferase (r = 0.44, P < 0.01).

HCV-RNA (β = 0.253; P < 0.05) were, in the order they entered into the model, independently associated with ALT levels.

Identification of a cut-off value for serum ALT levels according to gender

In order to establish a better cutoff value for ALT in hepatitis C screening in our Turkish population, the ALT levels were measured in 16 healthy age- and gender-matched volunteers. ALT levels in the control population were 18.2 ± 3.6 IU/L. The cutoff value was identified by construction of a ROC curve (receiver operating characteristic) in each gender. In females, the better cutoff value for ALT was at 22 IU/L, with sensitivity of 87.5% and specificity of 100% in identifying subjects without HCV infection (Figure 4). In males, the better cutoff value for ALT was at 22 IU/L, with sensitivity of 100% and specificity of 100% in identifying subjects without HCV infection (Figure 5).

DISCUSSION

This study provides insights into the correlates of ALT levels in the setting of patients with HCV infection. We found that, in our sample of Turkish patients, serum ALT levels were significantly and independently correlated with periportal bridging/necrosis, viral load and duration of HCV infection.

Serum ALT levels, as a measure of biochemical hepatitis activity, increased significantly with periportal bridging/necrosis, and this association was stronger than for other components of the HAI index. Our findings are in line with previous studies showing a statistically significant linear relationship between the degree of ALT elevation and the amount of liver injury based on the HAI score[17]. In our study, viral load was significantly and inversely correlated with mean ALT levels. This result is in keeping with the findings of Ito *et al*[18], who showed that mean viral load was significantly higher in chronic HCV patients with persistently normal ALT levels. In this regard, it has been hypothesized that immune response to HCV

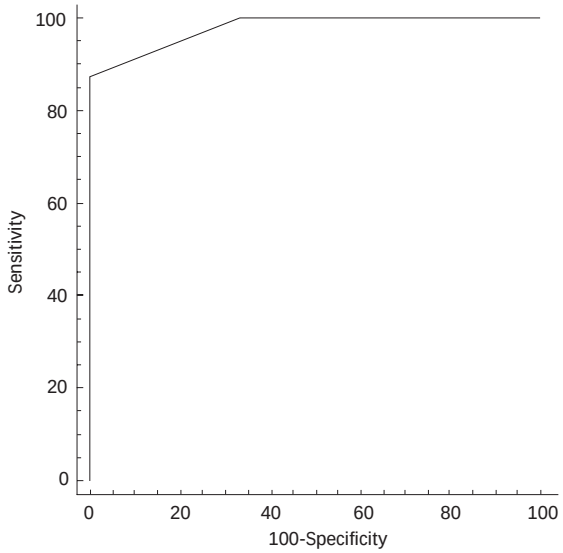

Figure 4 ROC curve of serum ALT for discriminating female HCV patients from women without liver disease. The better cutoff value for ALT was at 22 IU/L, with sensitivity of 87.5% and specificity of 100% in identifying subjects without HCV infection.

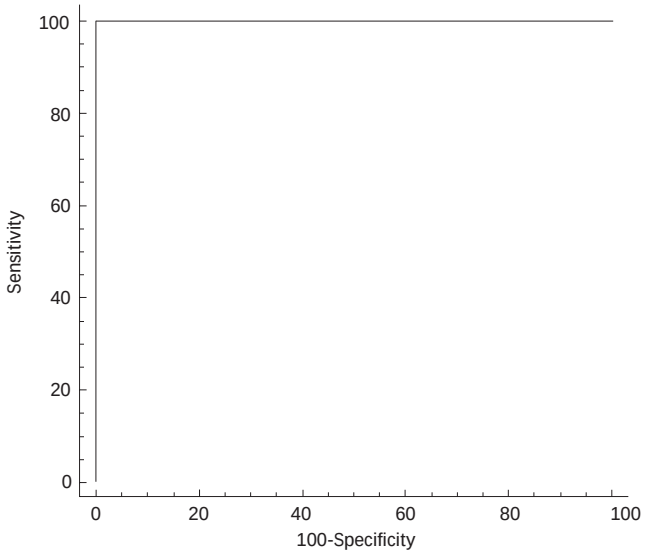

Figure 5 ROC curve of serum ALT for discriminating male HCV patients from men without liver disease. The better cutoff value for ALT was at 23 IU/L, with sensitivity of 100% and specificity of 100% in identifying subjects without HCV infection.

could play a role in rendering viral load smaller[18]. It should be noted, however, that some authors have reported higher ALT levels in patients with high viral load[19]. Another group has suggested that no significant difference in viral load exists between patients with abnormal ALT levels and those with normal ALT levels[20]. Although the reasons for conflicting data remain to be clarified, the discrepancies in the literature may be due at least in part to the presence of potential confounders such as ethnicity or different sample sizes. The duration of HCV infection may be of importance for the development of cirrhosis and patients with longer periods of infection may be more likely to have higher ALT levels[20]. In our study we found a positive association between ALT activity in the serum and duration of HCV infection. Some authors, however, have failed to demonstrate such a relationship[21]. In any case, it should be kept in mind that the onset of HCV infection may be difficult to establish in some patients, thereby rendering disease duration undefined.

Growing evidence has suggested that up to 25% of patients with chronic hepatitis C virus infection have persistently normal aminotransferase levels (10% to 40%, according to different studies)[22-24]. The normal range for ALT level was set in the 1950 s and has changed little since then. However, several recent studies have questioned whether previously established reference values to define normal ALT range are really accurate. Under these circumstances, it has been repeatedly suggested that the limits of normal ALT activity should be revised[25-27]. In most countries, the cutoff value for ALT is defined as twice the upper limit of the normal range of healthy individuals[28]. The normal ALT activity for men and women is < 23 IU/L and < 18 IU/L, respectively[28]. In order to gain more insights on the normal level of ALT in Turkish healthy adults at low risk for chronic liver diseases, we measured ALT activity in a control population from our country. By ROC curve analysis, we found that the optimal cutoff point in our population was 23 IU/L in males and 22 IU/L in females. Using our newly calculated cutoff value, we found that the sensitivity and specificity for detection of subjects without HCV infection were 100% and 100% in men and 87.5% and 100% in women, respectively. Hopefully, these new data obtained in the Turkish population should contribute to the ongoing discussion regarding new reference systems for the measurement of the catalytic activity of ALT in the clinical practice[29,30]. Given the small sample size, we believe that our findings may stimulate future studies on a larger number of patients.

In conclusion, we have provided evidence that the main correlates of ALT levels in HCV patients are periportal bridging/necrosis, viral load and duration of HCV infection. A cut-off value < 23 IU/L in males and 22 IU/L in females may distinguish with high diagnostic accuracy healthy control subjects from patients with HCV.

ACKNOWLEDGMENTS

The authors thank the assistance of the Research Fellows and the Scientific Staff at the Uludag University Medical School.

COMMENTS

Backgrounds

The exact definition of the normal levels of serum ALT activity is crucial for screening and follow-up studies in hepatitis C infection. However, half of the untreated patients with chronic HCV infections display normal or minimally elevated serum ALT levels. Accordingly, several studies have recently questioned whether previously established values to define normal ALT range are clinically accurate.

Research frontiers

Recent evidence has suggested that the limits of normal for serum ALT should be revised. In the present study, we sought to investigate serum ALT levels in relation to the clinical, biochemical, ultrasonographic and histological characteristics of patients with HCV.

Innovations and breakthroughs

We have provided evidence that the main correlates of ALT levels in HCV patients are periportal bridging/necrosis, viral load and duration of HCV infection.

Applications

A cut-off value < 23 IU/L in males and 22 IU/L in females may distinguish with high diagnostic accuracy healthy control subjects from patients with HCV.

Terminology

ALT elevation: aminotransferases leak from the liver into the blood when parenchymal liver cells are damaged.

Peer review

The study is of particular interest to the practical medicine. The results provide sufficient evidence that the activity of ALT correlates with histological changes, viral load and duration of infection in patients with HCV.

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RAPID COMMUNICATION

Risk factors of gastroesophageal reflux disease in Shiraz, southern Iran

Mehdi Saberi-Firoozi, Farnaz Khademolhosseini, Maryam Yousefi, Davood Mehrabani, Najaf Zare, Seyed Taghi Heydari

Mehdi Saberi-Firoozi, Farnaz Khademolhosseini, Maryam Yousefi, Davood Mehrabani, Seyed Taghi Heydari, Gastroenterohepatology Research Center/Department of Internal Medicine, Nemazee Hospital, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
Najaf Zare, Department of Biostatistics, School of Public Health, Shiraz University of Medical Sciences, Shiraz, Iran
Seyed Taghi Heydari, Department of Biostatistics, Lorestan University of Medical Sciences, Khoramabad, Iran
Correspondence to: Mehdi Saberi-Firoozi, MD, Associate Professor, Gastroenterohepatology Research Center, Nemazee Hospital, School of Medicine, Shiraz University of Medical Sciences, Karim-Khan Zand Blv, Shiraz, Iran. saberifm@sums.ac.ir
Telephone: +98-711-6276212 Fax: +98-711-6276212
Revised: December 21, 2006 Revised: July 28, 2007

Abstract

AIM: To determine the prevalence and symptoms of gastroesophageal reflux disease (GERD) in a healthy general population in relation to demographic, lifestyle and health-seeking behaviors in Shiraz, southern Iran.

METHODS: A total of 1978 subjects aged > 35 years who referred to Gastroenterohepatology Research Center and who completed a questionnaire consisting of 27 questions for GERD in relation to demographic, lifestyle and health-seeking behaviors were included in this study for a period of five months. The validity and reliability of the questionnaire were determined.

RESULTS: The prevalence of GERD was 15.4%, which was higher in females (17.3%), in rural areas (19.8%), and in illiterate subjects (21.5%) and those with a mean age of 50.25 years. The prevalence was significantly lower in subjects having fried food (14.8%), and fruit and vegetables (14.6%). More symptoms were noticed in subjects consuming pickles (22.1%), taking aspirin (21%) and in subjects with psychological distresses (27.2%) and headaches (22%). The correlation was statistically significant between GERD and halitosis (18.3%), dyspepsia (30.6%), anxiety (19.5%), nightmares (23.9%) and restlessness (18.5%). Their health seeking behavior showed that there was a significant restriction of diet (20%), consumption of herbal medicine (19%), using over-the-counter drugs (29.9%) and consulting with physicians (24.8%). Presence of GERD symptoms was also significantly related to a previous family history of the disease (22.3%).

CONCLUSION: GERD is more common in females, rural and illiterate subjects and correlated with consumption of pickles, occurrence of headache, psychological distress, dyspepsia, halitosis, anxiety, nightmare and restlessness, and a family history of GERD and aspirin intake, but the correlation was negative with consumption of fat and fiber intake.

Key words: Reflux; Risk factors; Prevalence; Southern Iran

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INTRODUCTION

Symptoms of gastroesophageal reflux disease (GERD) represent one of the most frequent health problems in the western world[1]. Approximately 10% of the American population suffer from daily heartburn and about one third have periodic symptoms[2]. Based on the population studied, the prevalence of the primary GERD symptoms, heartburn (a burning feeling behind the breast bone) or acid regurgitation (an acid taste in the mouth) varies between 9% and 42%[3]. The relationship between GERD and lifestyle habits, e.g. cigarette smoking, alcohol and coffee consumption, ingestion of medications such as aspirin and non-steroidal anti-inflammatory drugs (NSAIDs), and diet has not been firmly established, and inconsistent results have been obtained from population-based studies[1,4-8].

As there are few population-based data on GERD in Asia[9-12], this study was performed for the first time in Shiraz, southern Iran with the aim of determining the prevalence of GERD symptoms and describing the demographic, lifestyle and health-seeking behaviors associated with GERD.

MATERIALS AND METHODS

Materials

This study was carried out in a group of GERD patients.

Table 1 Frequency of GERD symptoms and their correlation with different characteristics of subjects in Shiraz, Southern Iran (*n* = 1978)

Characteristics		GERD Symptoms (%)		*P* value
		Present	Absent	
Gender	Male	12.3	87.7	0.003
	Female	17.3	82.7	
Habitat	Urban	13	87	0.001
	Rural	19.8	80.2	
	Illiterate	21.5	78.5	
Education	Primary school	16.1	83.9	< 0.001
	High school	13.6	86.4	
	University	12.3	87.7	
Physical activity	No	14.8	85.2	0.373
	Yes	16.3	83.7	
Psychological distress	No	14.9	85.1	0.003
	Yes	27.2	72.8	
Recurrent headache	No	14.7	85.3	0.009
	Yes	22	78	
Past GI disease history	No	13.3	86.7	< 0.001
	Yes	22.3	77.7	
	Thin	10.5	89.5	
Body mass index	Normal	15.7	84.3	0.065
	Overweight	13.4	86.6	
	Obese	18.8	81.2	
Age (mean) (yr)		50.25	49.83	0.547

GERD: Gastroesophageal reflux disease.

Methods

In a population-based study, 3600 subjects were selected by cluster random sampling method based on postal code division of Shiraz, southern Iran into 17 districts. After clarifying the research project for each subject, he/she received an invitation letter to refer to Mottahari Digestive Clinic of Gastroenterohepatology Research Center affiliated to Shiraz University of Medical Sciences. The project was approved by the Ethics Committee of the university and a written consent was obtained from each patient participating in the study. The study was undertaken for a period of five months from April to September 2004 while 1978 subjects completed the questionnaire. The included subjects were aged > 35 years, of both genders, and from both urban and rural areas. A team of interviewers who had received an intense training completed the questionnaire consisting of 27 questions categorized into three sections of demographic, lifestyle and symptoms of GERD (Appendix 1). A gastroenterologist completed the clinical questions of the questionnaire in the clinic. The reliability and validity of the questionnaire were determined by requesting 100 subjects to be interviewed at our clinic by the same trained interviewers and a gastroenterologist for completion of the questionnaire, respectively. Heartburn was defined as a burning feeling in epigastric area that rises through the chest in substernal area and acid regurgitation as liquid coming back into the mouth leaving a bitter or sour taste. A subject was defined to suffer from GERD when he/she reported heartburn and/or acid regurgitation in the preceding year with a frequency of at least three times a week irrespective of its severity or duration. Sociodemographic variables included age, gender, habitat, marital status, educational level, biological characteristics, such as BMI [weight in kg in the fasting state divided by the square of the height in meters, resulting in five categories of thin (< 18 kg/m^2), normal (18-24.9 kg/m^2), overweight (25-29.9 kg/m^2), obese (30-40 kg/m^2) and very obese (> 40 kg/m^2)], lifestyle such as physical activity (at least 30 min/week or sufficient to produce adequate sweating), dietary habits, cigarette smoking, alcohol, coffee and tea consumption and the use of aspirin and NSAIDs. Rural and urban habitats were defined by the size of the residence area (under 30 000 inhabitants *vs* 30 000 inhabitants or more). Dyspepsia was defined as epigastric or upper abdominal symptoms (pain or discomfort) in the past year. Information was put directly into a computer database under supervision of a professional biostatician.

Statistical analysis

Statistical analysis was performed using the SPSS computer software package (Version 11.5, Chicago, Il). A *P* value of 0.05 or less was considered to be statistically significant and all reported *P* values were two sided using Chi-square tests.

RESULTS

Among 3600 visited households, the interview questionnaire was completed in 1978 subjects (response rate, 54.9%; mean age, 49.90 ± 11.14 years). Among the subjects, 29.4% were male, 56.6 % lived in urban and 43.4% in rural regions; 39.7%, 29.7%, 17.2% and 13.5% of the subjects were respectively in 35-44, 45-54, 55-64 and > 65 years age groups; 25.6%, 32.3%, 14.5% and 27.6% of the participants were illiterate, or with primary, high school and university educational levels, respectively. The reliability and validity of the questionnaire were 82% and 70%, respectively.

The prevalence rate of GERD was 15.4% (304 subjects, GERD occurring at least 3 times per week). Table 1 shows the prevalence rates of GERD in relation to demographic data, revealing that the prevalence was higher in females (17.3%, $P = 0.003$), in rural areas (19.8%, $P = 0.001$), and in illiterate subjects (21.5%, $P = 0.001$). In subjects with GERD, a higher prevalence of psychological distress (27.2%, $P = 0.003$) and headaches (22%, $P = 0.009$) was observed.

Table 2 demonstrates the frequency of GERD symptoms in relation to dietary, smoking and drinking habits and medication of the participants. The results indicated a lower prevalence in subjects having fried food (14.8%, $P = 0.005$), and a higher prevalence among those consuming pickles (22.1%, $P = 0.001$). There was no association between GERD symptoms and drinking spirits ($P = 0.095$) or water ($P = 0.063$) with meals, salt intake ($P = 0.458$) and physical activity ($P = 0.373$). The correlation between GERD symptoms and subjects being a current or former cigarette smoker (10.8%, $P = 0.055$) or water pipe smoker (18.7%, $P = 0.096$) was not significant either.

The prevalence of GERD was lower in subjects with more fruits and vegetables intake (14.6%, $P = 0.001$) and those drinking tea (14.9%, $P = 0.465$) and coffee (12.9%, $P = 0.701$), but was higher among those drinking alcohol (15.6%, $P = 0.205$) but the difference was significant from those with consumption of fruits and vegetables.

Table 2 Prevalence of GERD in relation to lifestyle of subjects in Shiraz, southern Iran (n = 1978)

Life style and dietary habits		GERD symptoms (%)		*P* value
		Present	Absent	
Pickle	Yes	22.1	77.9	< 0.001
	No	12.8	87.2	
Salt	No	15	85	0.458
	Yes	16.3	83.7	
Fried food	No	24	76	0.005
	Yes	14.8	85.2	
Fast food	No	15.7	84.3	0.518
	Yes	14.5	85.5	
Fiber	No	30.2	69.8	< 0.001
(fruit and vegetables)	Yes	14.6	85.4	
Cigarette	No	15.9	84.1	0.055
	Yes	10.8	89.2	
Water pipe	No	14.8	85.2	0.096
	Yes	18.7	81.3	
Tea	No	16.1	83.9	0.465
	Yes	14.9	85.1	
Coffee	No	15.4	84.6	0.701
	Yes	12.9	87.1	
Spirit with meal	No	16.7	83.3	0.095
	Yes	14	86	
Water with meal	No	17.6	82.4	0.063
	Yes	14.3	85.7	
Alcohol	No	9.7	90.3	0.205
	Yes	15.6	84.4	
Feeding duration (min)	< 10	16.5	83.5	
	10-20	14.5	85.5	0.519
	> 20	16.1	83.9	
Aspirin	No	14.7	85.3	0.02
	Yes	21	79	
NSAIDs	No	14.5	85.5	0.067
	Yes	17.9	82.1	

GERD: Gastroesophageal reflux disease; NSAIDs: Non-steroidal anti-inflammatory drugs.

We noticed more symptoms in subjects taking NSAIDs (17.9%, $P = 0.067$), and aspirin (21%, $P = 0.020$) (Table 2), but the difference was only significant for aspirin. Subjects with GERD symptoms are restricting their diets (20%, $P = 0.001$), taking herbal medicine (19.0%, $P = 0.001$), using the over-the-counter (OTC) drugs (29.9%, $P = 0.001$) and consulting with physicians (24.8%, $P = 0.001$). In subjects consuming medication advised by their friends, the difference was not statistically significant (23.5%, $P = 0.058$). Subjects with GERD had a significantly higher accurrence of halitosis (18.3%; $P = 0.024$), dyspepsia (30.6%; $P = 0.001$), anxiety (19.5%; $P = 0.001$), nightmare (23.9%; $P = 0.001$) and restlessness (18.5%; $P = 0.001$) (Table 3). There was an association between GERD symptoms and a family history of the disease (22.3%; P=0.001) (Table 1).

Table 3 Health-seeking behavior of subjects with GERD Symptoms in Shiraz, Southern Iran (n = 1978)

Health-seeking behavior and associated symptoms		GERD Symptoms (%)		*P* value
		Present	Absent	
Restricting diet	No	12.9	87.1	< 0.001
	Yes	20	80	
Herbal medicine intake	No	13.2	86.8	0.001
	Yes	19	81	
Medication advised	No	15.1	84.9	0.058
by friends	Yes	23.5	76.5	
Over-the-counter drugs	No	12.2	87.8	< 0.001
	Yes	29.9	70.1	
Visiting physician	No	10.5	89.5	< 0.001
	Yes	24.8	75.2	
Halitosis	No	14.2	85.8	0.024
	Yes	18.3	81.7	
Dyspepsia	No	8.9	91.1	< 0.001
	Yes	30.6	69.4	
Anxiety	No	9.5	90.5	< 0.001
	Yes	19.5	80.5	
Nightmare	No	12.1	87.9	< 0.001
	Yes	23.9	76.1	
Restlessness	No	10.2	89.8	< 0.001
	Yes	18.5	81.5	

DISCUSSION

It has been estimated that the digestive disease with the highest annual direct cost in the USA is GERD (about US$ 9.3 billion)[13]. Furthermore GERD patients have reported decrements in the health-related quality of life when compared with the general population[14,15]. Among western patients, heartburn and acid regurgitation are known to be specific for GERD[16].

In our population-based study, the prevalence of GERD was 15.4% defined as heartburn and/or acid regurgitation at least three times per week. In a population-based study, Khoshbaten[17] reported a prevalence of 2.7% for GERD as heartburn occurring at least thrice in recent two weeks in Tabriz, northwestern Iran. This difference may be due to his different case definition in the questionnaire. In a sample of general population in Germany, 18% of subjects suffered from GERD[18]. Wong *et al*[15] in a study by telephone contact reported a prevalence of 29.8% in a Chinese population. A study by telephone calls in a Spanish population showed a prevalence rate of 25.2%-34.7%[19]. A monthly prevalence of 1.6% was reported in Singapore[9]. Hu *et al*[20] demonstrated that only 5% of a Chinese population had GERD. In a large study of Taiwanese patients, 17% had at least one of three reflux symptoms daily[21]. Several factors that may influence the prevalence of GERD have been identified, including genetic factors and differences in body mass index and lifestyle[22-25]. Geographical differences in GERD prevalence are difficult to interpret due to the different case definitions and questionnaires used[4,26].

As shown in Table 1, the GERD prevalence was higher in females, rural areas, and illiterate subjects and those with a mean age of 50.25 years. Wong *et al*[11], Diaz-Rubio *et al*[12] and Mahadeva *et al*[19] also reported a higher prevalence of GERD in females. Some studies have not demonstrated a relationship between gender and GERD[10,27]. In relation to habitat, Diaz-Rubio *et al*[19] showed that GERD prevalence was higher in rural areas and in relation to educational level, a higher prevalence in illiterate subjects similar to our study. The relationship between a lower educational level and the frequency of GERD was described previously, which probably reflects the action of certain unhealthy lifestyle habits, or less ability to modify such habits[7,19].

In relation to life style, smoking and alcohol have often been cited as risk factors for GERD, although findings of

studies on this matter have been inconsistent[3,27,28,29]. According to Nocon *et al*[18], smoking was a risk factor for GERD, which was associated with reflux symptoms and was dose-dependent. Nilsson *et al*[3], reported that smoking and salt were risk factors for reflux symptoms. Our results showed no correlation between GERD and smoking.

In our study, we found no effect of alcohol, tea, coffee and spirits on GERD symptoms. In a population-based study, Nilsson *et al*[3] did not notice any effect for these risk factors. No relationship in Nocon *et al's* study[18] was found between the intake of alcohol and reflux symptoms. Wong *et al*[11] and Mahadeva *et al*[12] indicated the increase of GERD prevalence due to consumption of alcohol, which is not consistent with our results. Drinks such as tea and coffee have also been reported to be linked to GERD but this is controversial. Although tea has been shown to increase gastric acid secretion, it does not appear to contribute to GERD[22]. Wendle *et al*[30] showed that coffee increases GERD and the irritant effect of coffee was correlated to the caffeine content, but this has also been disputed. Chang *et al*[21] found no link between coffee or tea consumption and the incidence of GERD. Diaz-Rubio *et al*[19] also noticed that occurrence of GERD was inversely associated with coffee consumption. There was also no effect of tea or coffee on GERD symptoms in Nocon *et al's* study[18]. The inverse relationship with coffee, tea or alcohol consumption should not be interpreted as a protective role for these beverages. Restriction in drinking of tea, coffee and alcohol may arise from the suggestions of their friends, even in our country, where alcohol consumption is not legally allowed. The role of coffee as a promoter of gastroesophageal reflux disease[30] is also consistent, suggesting that the avoidance of coffee is a sound recommendation for GERD sufferers. In relation to fiber intake, El-Serag *et al*[5] and Nocon *et al*[18] reported that consumption of fruits were associated with GERD symptoms and found a protective effect of dietary fiber, which was similar to our results. In relation to dietary fat, El-Serag *et al*[5] found an increased risk of GERD in subjects with a high intake of dietary fat. The data of Nocon *et al*[18] also showed that subjects with reflux symptoms tend to have a diet richer in fat. It has been shown that dietary fat can increase the transient lower esophageal sphincter relaxation[31], possibly *via* release of cholecystokinin[32]. Therefore, the lower fat content in a population may explain, in part, the lower prevalence of GERD.

In relation to consumption of spirits, we found no association between reflux symptoms and consumption of spirits. These results were different from Nocon *et al*[18]. With regard to physical exercise, there are conflicting results[29]. According to Nocon *et al*[18], subjects with GERD were less active physically, but our data did not confirm these results. Some studies have observed an association between the use of aspirin or NSAIDs and the presence of GERD[19,33] and use of NSAIDs is a risk factor for erosive esophagitis[5,15,34], whereas others have not[27,35]. A higher consumption of NSAIDs and aspirin were visible in subjects of our study with GERD symptoms but was only statistically significant for aspirin. In relation to medical care utilization, the results vary among countries from 16% to 56%. A study from Singapore found that 40% of GERD sufferers used OTC drugs or visited a physician for GERD symptoms[9]. This is in contrast with a study in Minnesota, USA, in which only 5.4% of GERD sufferers visited physicians[27]. In the study of Wong *et al*[15], 48% of subjects with GERD had received treatment, 6% had taken OTC medication, and 35% had visited physicians. In our study, in relation to health seeking behavior, there were significant differences between GERD symptoms and restricting diets, consumption of herbal medicine, using OTC drugs and visiting a physician. Caution should be taken when applying the data to countries in which medical care is available on a fee-for-service basis. Patients usually associate certain nutritional habits with the occurrence of reflux symptoms, and the avoidance of certain food is often cited as a therapeutic measure[1,7,8]. Nevertheless, the causal role of particular food in the etiology of GERD is still unclear. A family history of reflux symptoms was reported as a risk factor for GERD[36]. GERD in the sufferer's spouse or a direct family member was reported to be associated with the presence of GERD[19]. These results were identical to our data. In relation to BMI, although most studies have confirmed the association between BMI and GERD symptoms, the results to date have remained inconsistent. Risk factors for GERD in the West have been shown to include a high BMI[27]. Similar to our study, a cohort study from New Zealand, also found no association between BMI and reflux symptoms[37]. In contrast, the large population-based HUNT 2 study reported an association between BMI and reflux symptoms. Nocon *et al*[18] reported similar results while being overweight or obese was significantly associated with GERD symptoms. Hampel *et al*[38] also found a significant association between obesity and GERD symptoms. The association between obesity and the prevalence and severity of GERD was confirmed by several other authors[5,39].

Our study showed that halitosis, headaches, psychological distress, anxiety, nightmares and restlessnes were common in GERD subjects. The importance of psychological distress was also suggested by others[8,19,40]. Some population surveys conducted in western countries have suggested that patients with GERD have a higher level of stress and anxiety[14,40,41]. Wong *et al*[15] showed that psychological morbidity may play an important role in health care-seeking behavior, and co-existing depression and anxiety may act as a catalyst for a patient to seek medical care, rather than as a cause of symptoms. Lower levels of psychological well-being were observed in subjects with GERD[42]. The important strength of our study was its large sample of subjects in a healthy population, which is representative of the adult population in our country between the ages of 35-75 years.

In conclusion, the prevalence of GERD (15.4%) was significantly higher in females, rural and illiterate subjects. An inverse correlation was seen between GERD and consumption of fat and fiber intake. A correlation was noticed between GERD and pickle consumption, occurrence of headache, psychological distress, dyspepsia, halitosis, anxiety, nightmare and restlessness and a pervious family history of GERD. The association between GERD and aspirin was also significant. Future longitudinal studies and follow-ups are needed to clarify other possible risk factors and associations with GERD.

COMMENTS

Background

Symptoms of gastroesophageal reflux disease (GERD) represent one of the most frequent health problems in the western world. When compared with the general population, GERD patients have reported decrements in the health-related quality of life. Based on the population studied, the prevalence of the primary GERD symptoms, heartburn or acid regurgitation varies between 9% and 42%. The relationship between GERD and lifestyle habits, e.g. cigarette smoking, alcohol and coffee consumption, ingestion of medications such as aspirin and non-steroidal anti-inflammatory drugs (NSAIDs), and diet has not been firmly established, and inconsistent results have been obtained by population-based studies.

Research frontiers

The study was performed to determine the relationship between GERD and demographic factors, lifestyle habits, family history, health-seeking behaviors and other GI symptoms. How GERD might affect quality of life awaits further studies.

Innovations and breakthroughs

Many other studies on GERD were conducted by telephone surveys or in the form of questionnaires. In our study, however, subjects were interviewed face-to-face by a team of trained interviewers using a questionnaire for which validity and reliability had been determined. Both rural and urban inhabitants participated in this study, making possible a comparison of these two populations in regards to GERD. In our population-based study, the prevalence of GERD was 15.4%, which is higher than that reported by some other studies in Iran. Obesity, consumption of spirits and smoking have been suggested as the most important lifestyle risk factors for GERD symptoms, but we found no association between reflux symptoms and consumption of spirits, smoking or BMI.

Applications

The findings of this study are helpful to both the clinicians in handling GERD patients and patients in primary and secondary healthcare.

Terminology

Heartburn is defined as a burning pain or discomfort behind the breast bone. Acid regurgitation is referred to liquid coming back into the mouth leaving a bitter or sour taste.GERD, in this study, is considered as heartburn and/or acid regurgitation occurring at least three times per week.

Peer review

This is an extensive cross sectional study performed in Iran and gives attention to the contributing factors and demographics of GERD in the country of the authors.

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Appendix 1

Questionnaire
Gastroenterohepatology Research Center, Shiraz University of Medical Sciences
Frequency and associated factors of digestive and hepatic disorders in subjects aged ≥ 35 yr in Shiraz, Southern Iran

Questionnaire No: …….	Date: ……
Sex	Female □ Male □
Age	.....years
Marital status	Single □ Married □ Widow □ Divorced □
Habitat	Urban □ Rural □
Family size	.....
Education	Illiterate □ Primary □ Middle □ High school □ University □
Occupation	.....
Past medical history	Headache □ Psychological distress □ Hyperlipidemia □
Physical activity	.....Times per week
Family history of gastrointestinal diseases	Yes □ No □ If Yes : Specify the disease
Number of meals/day	Breakfast □ Lunch □ Dinner □ More □
Duration of serving each meal	.....min
Pickles consumption with meal?	Yes □ No □
Salt consumption with meal?	Yes □ No □
Having fast food?	Yes □ No □
If yes, how many/week?	.....
Having fried foods?	Yes □ No □
Any smoking?	Yes □ (Cigarette □ Water pipe □) No □
If yes	...../day .....year
Type of analgesics regularly used?	NSAIDs □ Aspirin □
Having fibers (fruits,vegetables)	Yes □ No □
Type and time of drinks?	Tea, after meal □ Water, with or after meal □ Coffee, after meal □ Spirit, with or after meal □
Alcohol drinking?	Yes, usually □ Yes, occasionally □ Never □
Any history of Gastroesophageal reflux (Heartburn or acid regurgitation) during last year?	Yes □ No □
Any upper abdominal discomfort or dyspepsia?	Yes □ No □
Health care-seeking behavior?	Diet restriction □ Herbal medicine□ Using medicine suggested by friends □ Over-the-counter drugs □ Visiting a physician □
Any complaints of:	Anxiety □ Nightmares □ Restlessness □ Halitosis □
Name and Signature of interviewer	……

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RAPID COMMUNICATION

A low prevalence of *H pylori* and endoscopic findings in HIV-positive Chinese patients with gastrointestinal symptoms

Fu-Jing Lv, Xiao-Lan Luo, Xin Meng, Rui Jin, Hui-Guo Ding, Shu-Tian Zhang

Fu-Jing Lv, Xiao-Lan Luo, Rui Jin, Hui-Guo Ding, Department of Digestive Diseases, Beijing You'an Hospital, Capital Medical University, Beijing 100069, China
Xin Meng, Pathology Department, Beijing You'an Hospital, Capital Medical University, Beijing 100069, China
Shu-Tian Zhang, Department of Digestive Diseases, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China
Correspondence to: Dr. Shu-Tian Zhang, Department of Digestive Diseases, Beijing Friendship Hospital, Capital Medical University, 95 Yong An Road, Xuanwu District, Beijing 100050, China. laolv2006@tom.com
Telephone: +86-10-63138339 Fax: +86-10-63020006
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Abstract

AIM: To compare the prevalence of *H pylori* infection, peptic ulcer, cytomegalovirus (CMV) infection and Candida esophagitis in human immunodeficiency virus (HIV)-positive and HIV-negative patients, and evaluate the impact of CD4 lymphocyte on *H pylori* and opportunistic infections.

METHODS: A total of 151 patients (122 HIV-positive and 29 HIV-negative) with gastrointestinal symptoms were examined by upper endoscopy and biopsy. Samples were assessed to determine the prevalence of *H pylori* infection, CMV, candida esophagitis and histologic chronic gastritis.

RESULTS: The prevalence of *H pylori* was less common in HIV-positive patients (22.1%) than in HIV-negative controls (44.8%; $P < 0.05$), and the prevalence of *H pylori* displayed a direct correlation with CD4 count stratification in HIV-positive patients. In comparison with HIV-negative group, HIV-positive patients had a lower incidence of peptic ulcer (20.7% *vs* 4.1%; $P < 0.01$), but a higher prevalence of chronic atrophy gastritis (6.9% *vs* 24.6%; $P < 0.05$), Candida esophagitis and CMV infection. Unlike HIV-negative group, *H pylori* infection had a close relationship to chronic active gastritis ($P < 0.05$). In HIV-positive patients, chronic active gastritis was not significantly different between those with *H pylori* infection and those without.

CONCLUSION: The lower prevalence of *H pylori* infection and peptic ulcer in HIV-positive patients with gastrointestinal symptoms suggests a different mechanism of peptic ulcerogenesis and a different role of *H pylori* infection in chronic active gastritis and peptic ulcer. The pathogen of chronic active gastritis in HIV-positive patients may be different from the general population that is closely related to *H pylori* infection.

Key words: Human immunodeficiency virus; Endoscopy; Cytomegalovirus; Candida esophagitis; *H pylori*; Peptic ulcer; Chronic gastritis

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INTRODUCTION

H pylori has been extensively studied and proven to be the main cause of chronic gastritis and peptic ulcer in the HIV-negative population[1,2]. The reported prevalence of *H pylori* in unselected populations ranges from 32% to 65%[3-6]. Over 90% patients with chronic active gastritis showed an evidence of *H pylori* infection[3,4,7], and 70%-100% of those patients had peptic ulcer disease[1,5,7].

In contrast, the prevalence of *H pylori* infection in patients infected with HIV has been reported to be remarkably low[8-11]. Reasons for these lower rates of *H pylori* infection remain unclear. Other studies showed that *H pylori* infection is similar in both HIV-positive and HIV-negative patients[12,13]. Patients infected with HIV, with or without acquired immune deficiency syndrome (AIDS), have a high incidence (50%-90%) of upper gastrointestinal symptoms[14]. The immune deficiencies caused by HIV give rise to many different gastrointestinal opportunistic infections, such as cytomegalovirus (CMV) infection and fungal esophagitis[15,16].

The aims of our study are to assess the prevalence of *H pylori* infection and the association with histological chronic active gastritis in HIV-positive patients with gastrointestinal symptoms. The impact of $CD4^+$ count on the prevalence of *H pylori*, gastric CMV infection and Candida esophagitis was also evaluated.

MATERIALS AND METHODS

Patients

The study was carried out at Beijing You'an Hospital,

Capital Medical University, Beijing, the largest referral center for management of HIV infection and HIV-related complications in China, from January 2003 to March 2006. Endoscopy was performed in 151 patients for gastrointestinal symptoms such as abdominal pain, dyspepsia, diarrhea, nausea, vomiting, gastrointestinal bleeding, and odynophagia or dysphagia.

The study groups consisted of 122 HIV-positive patients (49 males and 73 females; mean age 40.8 ± 7.9, range 26-60 years) and 29 age-matched HIV-negative patients (15 males and 14 females; mean age 49.5 ± 12.7, range 28-77 years) as control groups. The absolute $CD4^+$ lymphocyte count of HIV-positive patients at the time of endoscopic examination was measured with FACS Count Reagents (BD Company, USA). Patients all gave their consent before undergoing endoscopy, and the symptoms, consumption of medications within one month, including antibiotics, proton pump inhibitors were also recorded.

Endoscopy, diagnosis and histology

Video-endoscopes (Olympus XQ240, Tokyo, Japan) were used for the procedure. All patients underwent three biopsies from the lesser and greater curvature of the gastric antrum and lesser curvature of lower body, one for Rapid Urease Test (RUT) and two for histology. Additional biopsies were obtained from endoscopic lesions such as ulceration. The biopsy specimens were placed in 10% formaldehyde at the time of endoscopy and stained with hematoxylin-eosin, Warthin-Starry stains for histologic chronic gastritis and *H pylori* infection, and immunocytochemical techniques were performed for CMV infection (Monoclonal Mouse Anti-Human Cytomegalovirus, Dako). The *H pylori* infection was diagnosed by positive identification of both the organism on histology (Warthin-Starry) and RUT. The histologic gastritis was diagnosed according to the Sydney criteria[17]. Specimens were reviewed by only one pathologist who was blind to the status of those patients in present study.

The Candida esophagitis was diagnosed by sheathed brush cytology from endoscopic lesions, and gross appearance of mucosal presented with white plaques. Specimens obtained by sheathed brush should be smeared onto slides for fungi.

Statistical analysis

Chi-square test or Fisher exact probability tests were used to compare the prevalence of *H pylori*, CMV infection, Candida esophagitis, and peptic ulcer between HIV-positive patients, control groups, and HIV-infected patients with higher and lower $CD4^+$ counts and the use of antibiotics and proton pump inhibitors. Independent sample *t* test was used to compare the age and sex between the HIV-positive and control groups. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

The patient data and the prevalence of *H pylori* and endoscopic findings in HIV-positive patients and HIV-negative patients are shown in Table 1. The gastrointestinal symptoms of HIV-positive patients were mostly nonspecific, such as diarrhea, dyspepsia, abdominal pain, nausea, vomiting, and odynophagia or dysphagia. Only the occurrence of symptoms of diarrhea, odynophagia, and dysphagia in HIV-positive patients was significantly higher than that of control group ($P < 0.05$). The prevalence of *H pylori* infection was significantly lower in the HIV-positive group than that of HIV-negative control group (27/122; 22.1% *vs* 13/29; 44.8%, $P < 0.05$). Endoscopic examination revealed more patients with peptic ulcer in HIV-negative group than in HIV-positive group (6/29; 20.7% *vs* 5/122; 4.1%, $P < 0.01$). More histologic chronic atrophy gastritis was found in HIV-positive patients than in HIV-negative group (30/122; 24.6% *vs* 2/29; 6.9%, $P < 0.05$). Opportunistic infection by CMV was noted in 4.9% (6/122) HIV-positive patients but none in the HIV-negative group ($P = 0.49$). The incidence of Candida esophagitis in HIV-positive patients (19/122; 15.6%) was significantly higher than that of HIV-negative patients ($P < 0.05$).

H pylori infection was less common in those with $CD4^+$ counts < 200/μL than those with $CD4^+$ counts > 200/μL in HIV-positive patients (8/57; 14.0% *vs* 19/65; 29.2%, $P < 0.05$). Interestingly, the prevalence of *H Pylori* infection displayed a direct correlation with the $CD4^+$ lymphocyte count stratification in HIV-positive patients (Table 2). The Candida esophagitis was significantly more common in

Table 1 Patient data and clinicopathology

	HIV-positive (*n* = 122) (%)	HIV-negative (*n* = 29) (%)	*P*
Age (yr)	40.8 ± 7.9	49.5 ± 12.7	NS
Male	49	15	NS
Female	73	14	NS
Gastrointestinal symptoms			NS
Abdominal pain and distention	38	14	NS
Dyspepsia	42	6	NS
Diarrhea	27	1	0.02
Nausea and vomiting	47	8	NS
Odynophagia and dysphagia	21	0	0.035
Others	16	6	NS
Consumption of medications within one month, *n* (%)			
Antibiotics	47 (38.5)	3 (10.3)	0.004
Proton pump inhibitor	3 (2.5)	4 (13.8)	0.034
H pylori infection	27 (22.1)	13 (44.8)	0.013
Candida esophagitis	19 (15.6)	0 (0)	0.05
Peptic ulcer	5 (4.1)	6 (20.7)	0.007
Chronic atrophy gastritis	30 (24.6)	2 (6.9)	0.036
CMV infection	6 (4.9)	0 (0)	0.49

CMV: Cytomegalovirus; NS: Not significant.

Table 2 *H pylori* infection and previous use of antibiotics related to $CD4^+$ count in HIV-positive patients *n* (%)

$CD4^+$ count	*H pylori* infection	*P*	Antibiotic therapy	*P*
$CD4^+ \geq 200/\mu L$ (*n* = 65)	19 (29.2)	0.044	19 (29.3)	0.024
$CD4^+ < 200/\mu L$ (*n* = 57)	8 (14.0)		28 (49.1)	
$CD4^+ \geq 100/\mu L$ (*n* = 85)	24 (28.2)	0.014	29 (34.1)	0.13
$CD4^+ < 100/\mu L$ (*n* = 37)	3 (8.1)		18 (48.6)	

Table 3 Relationship of $CD4^+$ count to *H pylori* infection and Endoscopic Findings in HIV-positive patients *n* (%)

	$CD4^+ \geqslant 200/\mu L$ (*n* = 65)	$CD4^+ < 200/\mu L$ (*n* = 57)	*P*
H pylori infection	19 (29.2)	8 (14)	0.044
Candida esophagitis	4 (6.2)	15 (26.3)	0.002
Peptic ulcer	2 (3.1)	3 (5.3)	0.881
Chronic atrophy gastritis	13 (20)	17 (29.8)	0.209
CMV infection	1 (1.5)	5 (8.8)	0.155

Table 4 Relationship of chronic active gastritis to *H pylori* infection

	HIV-positive group (*n* = 122)		HIV-negative group (*n* = 29)	
	H pylori $^+$ (*n* = 27)	*H pylori* $^-$ (*n* = 95)	*H pylori* $^+$ (*n* = 13)	*H pylori* $^-$ (*n* = 16)
Chronic active gastritis, *n* (%)	8 (29.6)	16 (16.8)	8 (61.5)	1 (6.3)
P	0.140		0.005	

HIV-positive patients with $CD4^+$ count < 200/μL than those with $CD4^+$ count > 200/μL (15/57; 26.3% *vs* 4/65; 6.2%, $P < 0.01$), and the average $CD4^+$ counts of patients with Candida esophagitis was 116.47 ± 133.08/μL. The CMV infection was more common in HIV-positive patients with $CD4^+$ count < 200/μL than those with $CD4^+$ count > 200/μL , but it was not statistically significant (5/57; 8.8% *vs* 1/65; 1.5%, $P = 0.155$) (Table 3).

Histological examination revealed less chronic active gastritis in HIV-positive patients than in HIV-negative control group (24/122; 19.7% *vs* 9/29; 31%, *P* = NS), but the difference was not statistically significant. The relationship of *H pylori* infection with chronic active gastritis was evaluated in HIV-positive and HIV-negative patients (Table 4). In HIV-negative group, the incidence of chronic active gastritis was significantly higher in those with *H pylori* infection (61.5%) than those without (6.3%; $P < 0.01$). In HIV-positive group, the rate of chronic active gastritis was not significantly different between those with *H pylori* infection (29.6%) and those without (16.8%; $P = 0.14$).

The relationship in *H pylori* and CMV infection and peptic ulcer was evaluated between the two groups of patients. Peptic ulcer was detected in five HIV-positive patients, one of whom (20%) was positive for *H pylori* infection and one (20%) for CMV infection. In HIV-negative group, six patients were diagnosed as having peptic ulcer, four (67%) as *H pylori* infection and none as CMV infection.

The previous use of antibiotics and proton pump inhibitor was also evaluated between HIV-positive and HIV-negative patients (47/122; 38.5% *vs* 3/29; 10.3%, $P < 0.01$ and 3/122; 2.5% *vs* 4/29; 13.8%, $P < 0.05$, respectively) (Table 1). If $CD4^+$ count was taken into consideration, the use of all kinds of antibiotics in HIV-positive patients with $CD4^+$ counts < 100/μL was not significantly different in those with $CD4^+$ counts > 100/μL (18/37; 48.6% *vs* 29/85; 34.1%, $P = 0.13$) (Table 3). Those antibiotics mainly included Sulfonamides, penicillins and quinolones. None of the patients took NSAIDs, aspirin or steroid before endoscopic examination.

The HIV-positive patients in this study were usually concomitant with HCV and/or HBV infection which was more significantly frequent than in HIV-negative patients (102/122; 83.6% *vs* 2/29; 6.9%, $P < 0.01$). Nine patients with esophagogastric varices (7.4%) and 3 patients with portal hypertensive gastropathy (2.5%) in HIV-positive group were also found by endoscopic examination.

DISCUSSION

In the present study, we found that the majority of gastrointestinal symptoms of HIV-positive patients at our hospital were similar to that of HIV-negative group. In comparison with HIV-negative group, the symptoms of diarrhea, odynophagia, and dysphagia were significantly more in HIV-positive patients ($P < 0.05$). Several previous studies[16,18-19] revealed that more than 71% of AIDS patients who present with dysphagia and odynophagia have endoscopic evidence of esophageal candidiasis. Our result showed a high infection rate of Candida esophagitis in HIV-positive patients (19/122; 15.6%), which may be a possible explanation. Studies showed that the incidence of Cryptosporidium infection has been estimated to be 16%-33% in the AIDS patients in north America with chronic diarrhea[20,21]. In developing countries, the infection of Cryptosporidium was 55% among AIDS patients[22]. The etiological factor of diarrhea in HIV-positive patients in our study was not evaluated.

The prevalence of *H pylori* infection in HIV-positive patients at our hospital was significantly lower than that in HIV-negative control group. Our results were in agreement with some previous reports[8-11]. The reason of lower prevalence of *H pylori* infection may be lack of $CD4^+$ cells, use of antibiotics and proton pump inhibitor, decreased acid secretion, or competitive inhibition by other pathogens in HIV-positive patients.

According to previous reports, $CD4^+$ lymphocytes were reported to be involved[23-25] in the pathogenesis of *H pylori*-related gastritis or ulcer. It is well known that $CD4^+$ cells play a role in inducing gastritis and this gastritis might be a mechanism by which *H pylori* colonization is enhanced[26]. Patients with HIV infection and a low $CD4^+$ count would then lose this mechanism by which *H pylori* colonization is sustained, and infection intensity would diminish. In addition, the T-cell response to the organism could serve to induce tissue and epithelial damage. In AIDS patients, the decreased T-cell would induce a decreased incidence of *H pylori* gastritis[27]. In our results, a stratification of cases on the basis of $CD4^+$ count has shown a decrease of *H pylori* infection with the progression of HIV-related disease, and histological examination revealed less chronic active gastritis in HIV-positive patients than in HIV-negative control group (19.7% *vs* 31%). *H pylori* infection was closely related to chronic active gastritis in HIV-negative group ($P < 0.05$), but not in HIV-positive patients, indicating that other pathogens might exist , such as CMV and Cryptosporidium infection.

An impairment of *H pylori* colonization environment

might result from a progressive atrophic involution of the gastric mucosa with secondary decreased acid secretion in HIV-positive patients, which represents an altered intragastric environment[28,29]. In our results, histologic chronic atrophy gastritis in HIV-positive patients was significantly higher than in HIV-negative group (30/122; 24.6% *vs* 2/29; 6.9%, $P < 0.05$), which might be result of gastric secretory failure in HIV infection patients. The impaired acid secretion may allow subsequent gastric bacterial overgrowth and provide a less suitable environment or competitive inhibition for *H pylori* colonization.

An altered intragastric environment might also result from frequent use of antibiotics against apportunistic infections in patients at an advanced stage of HIV infection[30]. In comparison with HIV-negative group, HIV-positive group had a more frequent use of antibiotics. In HIV-positive patients, previous use of antibiotics with $CD4^+$ counts < 100/μL was not significantly different from that of $CD4^+$ counts > 100/μL ($P = 0.13$), but the prevalence of *H pylori* infection showed significant difference. In our patients, the antibiotics most frequently used was trimetoprim-sulfa, usually for treatment or prophylaxis against pneumocystis in HIV-positive patients, and monotherapy of antibiotics has been proven to inhibit rather than eradicate *H pylori*[31]. Therefore, current prophylaxis has been excluded from evaluation of eradicating the microorganism. Previous use of proton pump inhibitors might alter intragastric environment and therefore influence the prevalence of *H pylori* infection. In the present study, the HIV-negative control group took proton pump inhibitors more frequently than HIV-positive group (13.8% *vs* 2.5%, $P < 0.05$), which further proved the lower prevalence of *H pylori* infection in HIV-positive patients.

In this study, all 122 HIV-positive patients with gastrointestinal symptoms, only 4.1% had peptic ulcer, but 20.7% in HIV-negative group. This might explain that the low prevalence of *H pylori* infection result in the lower incidence of ulcers among HIV-positive patients. On the other hand, decreased acid secretion in HIV-positive patients plays a role in the lower incidence of peptic ulcer. According to previous reports, CMV-associated peptic ulcer disease was highly prevalent and CMV was the only organism significantly associated with gastroduodenal ulcers in HIV-positive patients, and *H pylori* was an uncommon cause of peptic ulcer[11,32]. In our study, among the 5 patients with peptic ulcer in HIV-positive group, only one proved to have CMV infection, which was lower according to previous studies[11,32]. The inadequate biopsies in the present study may be a possible explanation. According to literature, histological changes of CMV infection are patchy in distribution, however, and the single biopsy sensitivity for ulcerative lesions has been reported to be as low as 13%. Therefore, at least 8-10 biopsies of suspicious lesions are recommended[33].

Candida esophagitis is one of the most common opportunistic infections in patients with AIDS[34]. Our study showed that the Candida esophagitis was significantly higher in HIV-positive patients with $CD4^+$ count below 200/μL, and the average $CD4^+$ counts with Candida esophagitis was 116.47 ± 133.08/μL. According to previous studies, the CMV infection is also a common opportunistic pathogen in HIV-positive patients with a low $CD4^+$ count and is one of the main causes of gastrointestinal ulcer in AIDS patients[11,33]. The incidence of CMV infection in HIV-positive patients (4.9%) in our study was lower than previous reports[32]. The incorrect location of biopsy may be another possible reason. According to literature review, gastric CMV infections are usually seen in the fundus with contiguous involvement of the esophagus and gastroesophageal junction, and the distal stomach and antrum are less commonly involved[35,36]. In the present study, the biopsy specimens were usually obtained from gastric antrum and lower body, therefore might lower the incidence of CMV infection in our patients.

The HIV-positive patients in the present study, mainly from Henan Province of China, infected through illegal blood plasma collection, and usually coinfected with HCV and/or HBV infection (83.6%). Endoscopic examination also revealed findings such as esophagogastric varices and portal hypertensive gastropathy, which were significantly different from previous reports.

In summary, we have found that a lower prevalence of *H pylori* infection and peptic ulcer in HIV-positive patients with gastrointestinal symptoms than that of HIV-negative patients with similar symptoms. The mechanism of chronic active gastritis in HIV-positive patients may be different from HIV-negative group that was closely related to *H pylori* infection. Various opportunistic infections (especially Candida esophagitis) of upper gastrointestinal tract likely occur in HIV-positive patients with a $CD4^+$ count less than 200/μL.

COMMENTS

Background

Helicobacter pylori has been proven to be the main cause of chronic gastritis and peptic ulcer in the HIV-negative population. The role and prevalence of *H pylori* infection might be different in the HIV infected patients.

Research frontiers

The immune deficiencies caused by HIV give rise to many different gastrointestinal opportunistic infections, and the prevalence of *H pylori* infection in patients infected with HIV is remarkably low.

Innovations and breakthroughs

It is the first report to characterize the prevalence and role of *H pylori* infection in chronic active gastritis and peptic ulcer in HIV-positive patients infected through illegal blood plasma collection in China, who are usually coinfected with HCV and/or HBV. The pathogen of chronic active gastritis in HIV-positive patients may be different from the general population that was closely related to *H pylori* infection.

Applications

This observation might be of potential value in HIV-positive patients with gastrointestinal symptoms.

Peer review

The authors compared the prevalence of *H pylori* infection, peptic ulcer, cytomegalovirus (CMV) infection and Candida esophagitis in human immunodeficiency virus(HIV)-positive and HIV-negative patients. The lower prevalence of *H pylori* infection and peptic ulcer in HIV-positive patients with gastrointestinal symptoms suggests a different mechanism of peptic ulcerogenesis and a different role of *H pylori* infection in chronic active gastritis and peptic ulcer.

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RAPID COMMUNICATION

Effects of *H pylori* infection on gap-junctional intercellular communication and proliferation of gastric epithelial cells *in vitro*

Ran Tao, Miao-Feng Hu, Jin-Tu Lou, Yong-Liang Lei

Ran Tao, Miao-Feng Hu, Jin-Tu Lou, Yong-Liang Lei, Central Laboratory, Children's Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
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Correspondence to: Jin-Tu Lou, Central Laboratory, Children's Hospital, School of Medicine, Zhejiang University, 57 Zhugan Lane, Hangzhou 310003, Zhejiang Province, China. rtao1211@yahoo.com.cn
Telephone: +86-571-87061007-2426 Fax: +86-571-87033296
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Abstract

AIM: To explore the effects of *H pylori* infection on gap-junctional intercellular communication (GJIC) and proliferation of gastric epithelial cells *in vitro*.

METHODS: A human gastric epithelial cell line (SGC-7901) cultured on coverslips was exposed overnight to intact *H pylori* ($CagA^+$ or $CagA^-$ strains) and sonicated extracts, respectively. GJIC between the cells was detected by fluorescence redistribution after photobleaching (FRAP) technique. Proliferation of SGC cells was determined by methylthiazolyl tetrazolium (MTT) assay.

RESULTS: When compared with control in which cells were cultured with simple medium alone, both $CagA^+$ and $CagA^-$ *H pylori* isolates could inhibit GJIC ($CagA^+$: $F = 57.98$, $P < 0.01$; $CagA^-$: $F = 29.59$, $P < 0.01$) and proliferation ($CagA^+$: $F = 42.65$, $P < 0.01$; $CagA^-$: $F = 58.14$, $P < 0.01$) of SGC-7901 cells. Compared with $CagA^-$ strains, $CagA^+$ *H pylori* more significantly down-regulated GJIC of gastric cells (intact *H pylori*: $t = 13.86$, $P < 0.01$; sonicated extracts: $t = 11.87$, $P < 0.01$) and inhibited proliferation gastric cells to a lesser extent *in vitro* (intact *H pylori*: $t = 3.06$, $P < 0.05$; sonicated extracts: $t = 3.94$, $P < 0.01$).

CONCLUSION: Compared with $CagA^-$ *H pylori* strains, $CagA^+$ strains down-regulate GJIC of gastric epithelial cells more significantly and inhibit proliferation of gastric cells to a lesser extent *in vitro*. *H pylori*, especially $CagA^+$ strains, may play an important role in gastric carcinogenesis.

Key words: *H pylori*; Gap-junctional intercellular communication; Gastric epithelial cell; CagA; Fluorescence redistribution after photobleaching; Methylthiazolyl tetrazolium assay

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INTRODUCTION

Epidemiological and animal studies have demonstrated a strong causal relationship between gastric cancer and chronic infection with *H pylori,* especially cytotoxin-associated gene A (*cagA*)-positive strains[1,2]. The cagA gene product CagA is directly delivered into gastric epithelial cells *via* type Ⅳ secretion system. Following membrane localization and subsequent tyrosine phosphorylation, CagA interacts with a variety of host cell proteins that are involved in the regulation of cell growth and motility[3]. However, the exact mechanism responsible for the development of gastric cancer in *H pylori*-infected patients still remains unclear.

Gap-junctional intercellular communication (GJIC) is an important mechanism controlling cellular homeostasis, proliferation and differentiation. Inhibition of GJIC between adjacent cells has been postulated to be one of the important events occurring during the promotional stage of cancer[4]. The vast majority of neoplastic cells reduce GJIC compared to their nonneoplastic counterparts[5]. A number of tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), have been known as potent inhibitors of GJIC[6].

So far, changes of GJIC in *H pylori*-associated gastric carcinoma have not been extensively exploited. In the present study, we attempted to explore the molecular mechanisms of *H pylori* infection in gastric carcinogenesis by studying its effects on GJIC of gastric epithelial cells *in vitro*.

MATERIALS AND METHODS

H pylori strains

H pylori strains 97002 and 97004 were identified by and stored in Department of Medical Microbiology and

Table 1 Effects of intact *H pylori* and sonicated extracts on GJIC of SGC-7901 cells (*n*, mean ± SE)

Group	CagA$^+$ strain[b]	CagA$^-$ strain[b]
Intact *H pylori*[d]	26.05 ± 3.39 (40)[a]	36.95 ± 3.78 (44)[a]
Sonicated extracts[d]	15.92 ± 2.53 (40)[a]	22.69 ± 2.60 (41)[a]
Negetive control	66.39 ± 9.95 (24)	
Positive control (TPA)	8.47 ± 0.95 (22)	

[b]$P < 0.01$ one-way ANOVA *vs* negative control, [a]$P < 0.05$ ANOVA/Dunnett *vs* negative control, [d]$P < 0.01$ *vs* *t*-test of CagA$^+$ and CagA$^-$ *H pylori* strains.

Table 2 Effect of intact *H pylori* and sonicated extracts on proliferation of SGC-7901 cells (*n*, mean ± SE)

Group	CagA$^+$ strain[b]	CagA$^-$ strain[b]
Intact *H pylori*[d]	0.755 ± 0.048 (6)[a]	0.680 ± 0.036 (6)[a]
Sonicated extracts[d]	0.938 ± 0.037 (6)	0.830 ± 0.056 (6)[a]
Negetive control	0.955 ± 0.038 (6)	
Positive control (TPA)	0.986 ± 0.045 (6)	

[b]$P < 0.01$ one-way ANOVA *vs* negative control, [a]$P < 0.05$ ANOVA/Dunnett *vs* negative control, [d]$P < 0.01$ *vs* *t*-test of CagA$^+$ and CagA$^-$ *H pylori* strains.

Parasitology, Zhejiang University School of Medicine. The genotypes of vacuolating cytotoxin gene A (*vacA*) of the strains 97002 and 97004 were s1a/m1 and m2, respectively. The results of Western blot and cell vacuolation test demonstrated that the strain 97002 was CagA$^+$/VacA$^+$ and 97004 CagA$^-$/VacA$^-$.

H pylori culture

H pylori strains were cultured on ECY blood-free medium[7] at 37℃ for 5 d, under 100% humidity and microaerophilic conditions (50 mL/L O_2, 100 mL/L CO_2, and 850 mL/L N_2). The bacteria were harvested from the agar plates, washed twice with 0.01 mol/L PBS and stored at -20℃.

Preparation of intact H pylori and sonicated extract samples

The frozen bacteria were dissolved in RPMI1640 culture medium and adjusted to 1×10^{10} CFU/L in intact bacterial samples and 1×10^{12} CFU/L in sonicated extract samples, respectively. The preparation of sonicated extract samples additionally included *H pylori* pulverization with ultrasound, centrifugation at 10000 r/min for 20 min with the supernatant collected.

Cell culture

Human gastric epithelial cell line SGC-7901 was obtained from Department of Medical Microbiology and Parasitology, Zhejiang University School of Medicine and cultured in RPMI1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, China), 1×10^5 IU/L penicillin and 100 mg/L streptomycin. The cells were incubated at 37℃ in a humidified atmosphere containing 950 mL/L air and 50 mL/L CO_2. The cells were grown on 22 mm × 22 mm coverslips in tissue culture dishes (35 mm in diameter) and the culture medium was changed every other day. To determine cell proliferation, SGC-7901 cells were plated into 96-well microplates (0.5×10^5 cells/well) and cultured for 12 h.

Cell treatment with H pylori extracts

Twenty-four hours prior to GJIC measurement, cells of the test groups were treated overnight with intact *H pylori* or sonicated extracts. Negative and positive controls were treated with RPMI1640 with 2% NBS and 5 μg/L TPA was added to the positive control during the last 1 h.

Measurement of GJIC by FRAP technique

GJIC between SGC-7901 cells was measured by fluorescence redistribution after photobleaching (FRAP) technique first described in 1986[8]. 6-carboxyfluorescein diacetate (6-CFDA) was used as the dye that could be retained inside the cells due to its hydrolysis by cytoplamic esterases into 6-carboxyfluorescein (6-CF). 6-CF could permeate gap junction channels due to its low molecular weight. FRAP was achieved under a confocal laser scanning microscope (Leica TCS-SP, Germany) and the detailed protocol was performed as previously described[9].

Determination of cell proliferation by MTT assay

When SGC-7901 cells confluenced by 70% in the 96-well microplates, cell proliferation was assessed by methylthiazolyl tetrazolium (MTT) assay as previously described[10]. The absorbance value per well at 570 nm was read on an automatic multiwell spectrophotometer (Bio-Rad, USA).

Statistical analysis

All data were presented as mean ± SE. Statistical analysis was carried out by ANOVA followed by Dunnett's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

H pylori down-regulated GJIC of SGC-7901 cells

The GJIC of SGC-7901 cells was measured by FRAP after treated with intact *H pylori* or sonicated extracts for 24 h and presented as fluorescence transfer rate (K, 10^{-3}/s) (Table 1). In the present study, both CagA$^+$ and CagA$^-$ *H pylori* isolates including intact *H pylori* and sonicated extracts down-regulated GJIC of SGC-7901 cells (CagA$^+$: $F = 57.98$, $P < 0.01$; CagA$^-$: $F = 29.59$, $P < 0.01$). Compared with CagA$^-$ strains, CagA$^+$ *H pylori* more significantly down-regulated GJIC of gastric cells (intact *H pylori*: $t = 13.86$, $P < 0.01$; sonicated extracts: $t = 11.87$, $P < 0.01$). In addition, our study demonstrated that TPA (5 μg/L for 1 h) had a significant inhibitory effect on GJIC of gastric cells.

Effect of H pylori on cell proliferation

The effects of intact *H pylori* and sonicated extracts on the proliferation of SGC-7901 cells were evaluated by MTT assay ($A_{570\ nm}$) (Table 2). The results suggest that both CagA$^+$ and CagA$^-$ *H pylori* isolates inhibited proliferation of SGC-7901 cells (CagA$^+$: $F = 42.65$, $P < 0.01$; CagA$^-$: $F = 58.14$, $P < 0.01$). However, CagA$^+$ *H pylori* strain inhibited proliferation of gastric cells to a lesser extent when compared with CagA$^-$ strain (intact *H pylori*: $t = 3.06$, $P < 0.05$; sonicated extracts: $t = 3.94$, $P < 0.01$).

DISCUSSION

Among various forms of intercellular communication systems in multicellular organisms, GJIC is the only form by which cells exchange signals directly from the inside of one cell to the neighboring cells. GJIC plays a crucial role in maintaining homeostasis by keeping growth control signals at equilibrium among GJIC-connected cells[11,12]. Most tumor cells have a reduced ability to communicate among themselves and/or with surrounding normal cells, confirming the importance of functional GJIC in growth control[13-15]. GJIC is mediated by gap junction channels composed of tetramembrane spanning proteins, known as connexins. At least 13 subtypes of connexin have been identified and four or five subtypes are detectable in the gastrointestinal tract[16].

It has been reported that connexin 32 in normal gastric mucosa is reduced significantly or absent in atrophic gastric mucosa and metaplastic epithelial cells, and no malignant cells from patients with gastric carcinoma contain detectable connexin 32[17,18]. These results suggest that loss of cell-cell communication through the gap junction may act as an early indicator of gastric carcinoma.

In this study, the effects of *H pylori* infection on GJIC of gastric epithelial cells were detected *in vitro*, suppressing interferences of various cytokines and immune factors *in vivo*, suggesting that both $CagA^+$ and $CagA^-$ *H pylori* isolates inhibit GJIC of SGC-7901 cells and the down-regulating effect of $CagA^+$ *H pylori* is more significant than that of $CagA^-$ strains. These findings emphasize the close relationship between *H pylori* especially $CagA^+$ strains and gastric carcinoma.

Increased cellular proliferation rates are characteristic in malignant tissue. Because of unstability of the genome of proliferating cells, hyperproliferation increases the possibility of DNA damage and aneupoidy. Dysplasia may evolve into carcinoma if damaged DNA cannot be repaired on time or fails in promoting the apoptosis system[19]. *H pylori* infection of the gastric mucosa is closely associated with changes in gastric epithelial cell proliferation. *In vivo* data show that gastric epithelial hyperproliferation is common in *H pylori*-infected persons and the degree of proliferation is directly associated with the severity of mucosal neutrophilic infiltration[20-22]. However, it was reported that an overall increase in gastric epithelial cell proliferation is not associated with *H pylori* gastritis[23]. It is not very clear whether the increased proliferation seen *in vivo* is a direct effect of *H pylori*, or a reflex increase in proliferation in response to increased cell damage, indirectly caused by *H pylori*. A recent report by Cabral *et al*[24] suggested that the increased cell proliferation rate in patients with *H pylori* infection might be related to the *H pylori*-induced inflammation rather than to a direct action of the pathogen.

Several *in vitro* studies reported that *H pylori* can inhibit cell proliferation[25,26] , which is consistent with the results of this study. The possible reason for the contradiction between the findings *in vivo* and *in vitro* is that *in vivo* studies are representative of the effect of persistent *H pylori* infection whereas *in vitro* experimental studies are representative of an acute *H pylori*-mediated effect. Also, the increased cell proliferation in patients with *H pylori* infection might be due to the increased production of gastrin *in vivo*[25]. Moreover, *in vivo* increased epithelial cell injury is associated with a reflex increase in proliferation of uninjured cells, which would not be seen *in vitro* as each cultured gastric cell is in contact with bacteria[27]. Cell proliferation is an essential process for the integrity of gastric mucosa. Deceasing cell turnover may increase the chances of ulcer formation and delay ulcer healing. Therefore, our findings seem to be relevant to the pathogenesis of *H pylori*-associated peptic ulcer diseases.

$CagA^+$ *H pylori* is frequently isolated from patients with gastric cancer in Western countries and may be more virulent in its pathogenesis[28,29]. *In vivo* studies reported that infection with $CagA^+$ *H pylori* strains is linked with higher acute inflammatory scores than $CagA^-$ strains[30,31], suggesting that these strains preferentially induce epithelial cell proliferation by stimulating inflammatory mediators. Our results show that $CagA^+$ strains could inhibit proliferation of gastric epithelial cells to a lesser extent than $CagA^-$ ones. Thus gastric cells injured by exposure to $CagA^+$ *H pylori* strains may be more likely to progress through the cell cycle, which possibly results in the risk of replication of cells with DNA damage[27].

In conclusion, *H pylori* can directly inhibit GJIC and proliferation of gastric epithelial cells *in vitro*. Compared with $CagA^-$ *H pylori* strains, $CagA^+$ strains more significantly down-regulate GJIC and inhibit proliferation to a of gastric epithelial cells lesser extent. Accelerated proliferation increases the risk of DNA damage and gene mutation. Inhibited GJIC makes cancer-initiated cells escape from the control of neighboring cells. *H pylori*, especially $CagA^+$ strains, may play an important role in gastric carcinogenesis.

COMMENTS

Background

It has been widely accepted that there is a strong association between *H pylori* infection and gastric cancer, but the exact molecular mechanism of the pathogen in gastric carcinogenesis has not clarified yet. Nearly 40 years ago, loss of functional gap junctions was described in cancer cells and led to the hypothesis that such a type of intercellular communication is involved in the carcinogenesis process. Since then, a lot of data have been accumulated confirming that gap junctions are frequently decreased or absent in cancer cells. Gap junction deficiency has been defined in the literature either as the lack of gap-junction plaques or as the lack of gap-junctional intercellular communication (GJIC). It has been reported that connexin 32 in normal gastric mucosa as a mediator of GJIC is reduced significantly or absent in atrophic gastric mucosa and metaplastic epithelial cells. However, these reports have not revealed the relationship between changed GJIC and *H pylori* infection of the gastric mucosa.

Research frontiers

There has been a considerable interest over recent years in factors that predispose individuals to develop gastric carcinoma. Complex interactions between several *H pylori*, host genetics and environmental factors determine this predisposition. Understanding the molecular mechanism of the interaction between *H pylori* and gastric epithelial cells will provide us with a new strategy for effective prevention of the development of gastric cancer induced by *H pylori* infection.

Innovations and breakthroughs

In this article, the molecular mechanism of *H pylori* infection in gastric carcinogenesis was explored by studying its effects on GJIC of gastric epithelial cells *in vitro*. The results suggest that *H pylori* could inhibit GJIC of cultured gastric epithelial cells and the down-regulation effect on GJIC of $CagA^+$ strains was more significant than $CagA^-$ ones.

Applications

This article emphasizes the close relationship between *H pylori* especially $CagA^+$ strains and gastric carcinoma. It provides a new direction to illuminate the molecular mechanism of *H pylori* in gastric carcinogenesis. It also implies that compounds able to restore GJIC in junctional deficient cells or prevent its disruption in junctional proficient cells may be used in making new strategies for the prevention and/or treatment of human gastric malignancies.

Terminology

Gap junctions: membrane structures made of intercellular channels which permit the diffusion of small hydrophilic molecules from cytoplasm to cytoplasm.

Peer review

The paper seems innovative. Altered expressions of connexins have been observed in various pathological processes of the digestive tract, including gastric cancer. To our knowledge, it is the first study to explore the molecular mechanism of *H pylori* infection in gastric carcinogenesis by studying its effects on GJIC of gastric epithelial cells *in vitro*.

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RAPID COMMUNICATION

Regulation of activin receptor-interacting protein 2 expression in mouse hepatoma Hepa1-6 cells and its relationship with collagen type Ⅳ

Hong-Jun Zhang, Gui-Xiang Tai, Jing Zhou, Di Ma, Zhong-Hui Liu

Hong-Jun Zhang, Gui-Xiang Tai, Jing Zhou, Di Ma, Zhong-Hui Liu, Department of Immunology, School of Basic Medical Sciences, Jilin University, Changchun 130021, Jilin Province, China
Supported by the National Natural Science Foundation of China, No. 30170478 and 30571688, and Science Projects of Jilin Province of China, No. 20060928-01
Correspondence to: Professor Zhong-Hui Liu, Department of Immunology, School of Basic Medical Sciences, Jilin University, Changchun 130021, Jilin Province, China. zh_liu2001@yahoo.com.cn
Telephone: +86-431-85619476 Fax: +86-431-85639362
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Abstract

AIM: To investigate the regulation of activin receptor-interacting protein 2 (ARIP2) expression and its possible relationships with collagen type Ⅳ (collagen Ⅳ) in mouse hepatoma cell line Hepal-6 cells.

METHODS: The ARIP2 mRNA expression kinetics in Hepal-6 cells was detected by RT-PCR, and its regulation factors were analyzed by treatment with signal transduction activators such as phorbol 12-myristate 13-acetate (PMA), forskolin and A23187. After pcDNA3-ARIP2 was transfected into Hepal-6 cells, the effects of ARIP2 overexpression on activin type Ⅱ receptor (ActRⅡ) and collagen Ⅳ expression were evaluated.

RESULTS: The expression levels of ARIP2 mRNA in Hapel-6 cells were elevated in time-dependent manner 12 h after treatment with activin A and endotoxin LPS, but not changed evidently in the early stage of stimulation (2 or 4 h). The ARIP2 mRNA expression was increased after stimulated with signal transduction activators such as PMA and forskolin in Hepal-6 cells, whereas decreased after treatment with A23187 (25.3% ± 5.7% *vs* 48.1% ± 3.6%, $P < 0.01$). ARIP2 overexpression could remarkably suppress the expression of ActRIIA mRNA in dose-dependent manner, but has no effect on ActRⅡB in Hepal-6 cells induced by activin A. Furthermore, we have found that overexpression of ARIP2 could inhibit collagen Ⅳ mRNA and protein expressions induced by activin A in Hapel-6 cells.

CONCLUSION: These findings suggest that ARIP2 expression can be influenced by various factors. ARIP2 may participate in the negative feedback regulation of signal transduction in the late stage by affecting the expression of ActRIIA and play an important role in regulation of development of liver fibrosis induced by activin.

Key words: Activin receptor-interacting protein 2; Hepal-6 cells; Lipopolysaccharide; Phorbol 12-myristate 13-acetate; Forskolin; Collagen

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INTRODUCTION

Activin is a multifunctional growth and differentiation factor of transforming growth factor-beta (TGF-β) superfamily[1,2]. As an important regulator, activin is involved in the acute phase response of inflammatory diseases and tissue repair, and also play an important role in inducing liver fibrosis[3-5]. The actions of activin on target cells are tissue-specific, which associate with the difference of activin receptor signal transduction. It has been found that the tissue-specificity might depend on a new group of intracellular signal proteins, activin receptor-interacting proteins (ARIPs)[6-8]. ARIPs have four forms at least, all of which can specifically interact with activin type Ⅱ receptor (ActRII) and regulate intracellular signal transduction induced by activin[6-10]. It has been demonstrated that not only the expression and distribution but also the biological activities of ARIPs were obviously different in various tissues. ARIP2 can enhance ActRII endocytosis and reduce ActRIIA receptor expression on cell membranes *via* Ral/RalBP1-depending pathway, and has a capability of suppressing activin-induced signal transduction. There was high expression of ARIP2 mRNA in liver tissues tested by Northern blot[7]. Therefore, we reason out that ARIP2 may participate in the functional regulation of hepatocytes treated by activin.

Since ARIP2 has only been recently discovered, the mode of expression regulation and function of it have not been well characterized. In this study, we have explored

regulation of ARIP2 expression and its effects on the expression of collagen type Ⅳ (collagen Ⅳ) which is component of extracellular matrix (ECM), using mouse Hepal-6 cells, which were obtained from mouse hepatoma cell line and had functions of hepatic parenchymal cells[11].

MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS, from E.coli 0111:B4), A23187, phorbol 12-myristate 13-acetate (PMA) and forskolin were obtained from Sigma. AMV Reverse Transcriptase was purchased from Promega. ExTaq was obtained from Takara Biotechnology Co (Kyoto, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO. Trizol reagent was obtained from Invitrogen. Activin A was provided by Dr. Eto T (Ajinomoto Central Research Laboratories, Japan).

Cell culture

Hepa1-6 cells from mouse hepatoma cell line were provided by Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and were maintained in DMEM medium supplemented with 10% fetal calf serum (FCS) at 37℃ in a 5% CO_2 humidified incubator.

Plasmid construction

The vector construction has been described previously[8]. The set of primers was designed as follows. The sense primer was 5'-GGAATTCATGAACGGACGGGTGGATTA-3', which introduced an *EcoR* I site, and the anti-sense primer 5'-GCTCGAGTCATTGTCTGCACAATAAACA-3', which introduced an *Xhol* I site. cDNA fragments encoding full-length ARIP2 (1-153 amino acid residues) were amplified by PCR. The amplified cDNA fragments were inserted into plasmid pMD18-T, and were then subcloned into eukaryotic expression vector pcDNA3. The reconstructed plasmid was named as pcDNA3-ARIP2.

Detection of ARIP2 mRNA expression in Hepal-6 cells stimulated by activin A and LPS

Hepal-6 cells were plated into 12-well tissue culture plates at a density of 2×10^5 cells/mL and incubated in 10% FCS-DMEM at 37℃, 5% CO_2 over night. The cells were cultured in 2% FCS-DMEM in the presence or absence of activin A (5 ng/mL) and LPS (2.5 μg/mL), respectively. After 2, 4, 8, 12 and 24 h, the cells were harvested respectively and total RNA was extracted by using the TRIzol reagent according to the manufacturer's protocol (Invitrogen). The mRNA expression of ARIP2 was examined by RT-PCR, and GAPDH was considered as inner control. PCR was performed for 30 cycles. Amplified PCR products were subjected to 1.5% agarose gel electrophoresis, and stained with ethidium bromide for detection. Specific bands were analyzed using ImageMaster VDS (Pharmacia Biotech Company, Sweden). The primer sequences were shown at Table 1.

Assay of the effects of signal transduction kinetins on the expression of ARIP2 mRNA

To further study the regulation elements of ARIP2 expression, the Hepal-6 cells plated into 12-well tissue culture plates were cultured in 2% FCS-DMEM in the presence or absence of activin A (5 ng/mL), A23187 (200 nmol/L), PMA (20 nmol/L), forskolin (50 μmol/L) and LPS (2.5 μg/mL), respectively. After 24 h, the cells were harvested respectively and total RNA was extracted by using the TRIzol reagent. The expression of ARIP2 mRNA was examined by RT-PCR.

Overexpression of ARIP2 in Hepa1-6 cells

To determine possible bioactivity of ARIP2, effects of ARIP2 on the mRNA expressions of ActRIIA, ActRIIB and collagen type Ⅳ were analyzed by RT-PCR. The Hepal-6 cells were washed once with serum-free DMEM, and were then transfected with pcDNA3-ARIP2 (0.1, 0.3 μg) and pcDNA3 (0.3 μg) by using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen), respectively. The transfected cells were incubated in the presence or absence of activin A (5 ng/mL) overnight. The cultured cells were harvested and total RNA was extracted by using the TRIzol reagent. RT-PCR was performed for detecting ActRIIA, ActRIIB and type Ⅳ collagen mRNA expressions. The primer sequences were shown at Table 1.

Flow cytometry for type Ⅳ collagen protein expression

Hepal-6 cells were collected 24 h after transfected with pcDNA3-ARIP2. The expression of type Ⅳ collagen proteins were assessed by flow cytometry (FACSort Vantage; BD, Franklin Lakes, NJ) using anti-mouse type Ⅳ collagen antibodies. The data were collected and analyzed on computer (Cell Quest software; BD Biosciences), to assess the percentage of positive fluorescence cells. A representative experiment of the two performed was shown.

RESULTS

Kinetics of ARIP2 mRNA expression in Hepa1-6 cells stimulated by activin A and LPS

As a regulation protein of activin signal pathway, the expression of ARIP2 mRNA could be increased by stimulation with activin A. In this study, the levels of ARIP2 mRNA expression were time-dependently up-regulated 12 h after treatment with activin A in Hepal-6 cells, but not obviously changed at 2-4 h after being treated with activin A. Endotoxin LPS as inflammatory factor can bind with Toll-like receptor 4 on hepatocytes. We found that ARIP2 mRNA expression in Hepal-6 cells was remarkably promoted by LPS treatment, and the expression levels were time-dependently up-regulated 12 h after treatment with LPS in Hepal-6 cells (Figure 1). These data suggested that the expression of ARIP2 was increased in the late stage of activin A and LPS treatment, and ARIP2 might participate in the negative regulation of the late stage signal transduction in Hepal-6 cells.

The signal transduction kinetins regulated the expression of ARIP2 mRNA

PMA is the activator of protein kinase C (PKC)[12], A23187 is the calcium ion vector[13], forskolin is the kinetin of cAMP-dependent protein kinase A (PKA)[14]

Table 1 Primer sequences used in transcriptase-polymerase chain reaction (PCR)

Target	Primers	Sequences	Products size (bp)	Genbank No.
GAPDH	Sense	5′-GATTGTTGCCATCAACGACC-3′	371	BC083149
	Antisense	5′-GTGCAGGATGCATTGCTGAC-3′		
ARIP2	Sense	5′-GTCAGCCGTATCAAAGAGGATG-3′	371	AY157057
	Antisense	5′-CTTGTGGCAATACTTCTCTGGTG-3′		
ActRIIA	Sense	5′-ATTGGCCAGCATCCATCTCTTG-3′	296	XM_123799
	Antisense	5′-TGCCACCATCATAGACTAGATTC-3′		
ActRIIB	Sense	5′-TGCTGAAGAGCGACCTCAC-3′	544	NM_007397
	Antisense	5′-AGCAGGTCCACATTGGTGAC-3′		
Collagen Ⅳ	Sense	5′-GCCTGCTCAAGGAGAAGACA-3′	380	NM_007734
	Antisense	5′-GATCCATAGGAGTCTCCAGGT-3′		

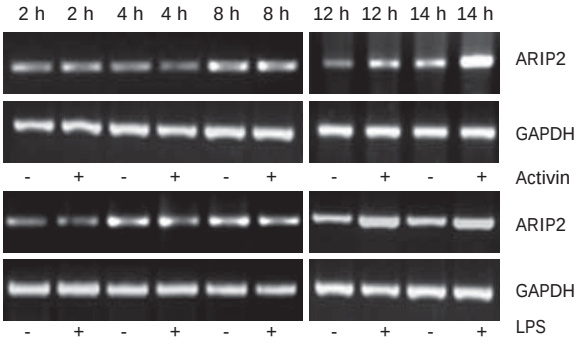

Figure 1 Expressions of ARIP2 mRNA in Hepal-6 cells stimulated by activin A and LPS.

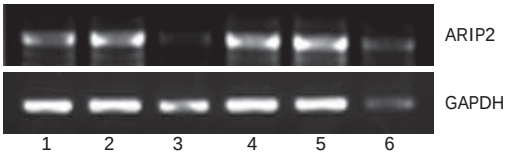

Figure 2 The effects of signal transduction activators on expressions of ARIP2 mRNA. Lane 1: Hepal-6 cells untreated; Lane 2: Treated with activin A (5 ng/mL); Lane 3: A23187 (200 nmol/L); Lane 4: LPS (2.5 μg /mL); Lane 5: PMA (20 nmol/L); Lane 6: forskolin (50 μmol/L).

and endotoxin LPS can bind with Toll-like receptor 4 on the surface of hepatocytes to stimulate cellular activities non-specifically[15]. To further study the regulation factors of ARIP2 expression, we used all of the above signal transduction activators to stimulate Hepal-6 cells and observed the expression of ARIP2 mRNA. The results showed that activin A (ARIP2 mRNA content relative to GAPDH, 66.2% ± 4.9%), LPS (76.5% ± 5.7%), PMA (72.3% ± 5.2%) and forskolin (79.8% ± 6.6%) could promote the expressions of ARIP2 mRNA (untreated control group, 48.1% ± 3.6%), whereas A23187 (25.3% ± 5.7%) could suppress it markedly (Figure 2), 25.3% ± 5.7% *vs* 48.1% ± 3.6%, $P < 0.01$. These data indicated that activators of the PKC, PKA signal transduction pathways and LPS *via* Toll-like receptor 4 could up-regulated the expression of ARIP2 mRNA.

Effects of ARIP2 overexpression on ActRII expression in Hepal-6 cells

To investigate the biological activities of ARIP2 expression in Hepal-6 cells, expression vectors pcDNA3-ARIP2 were

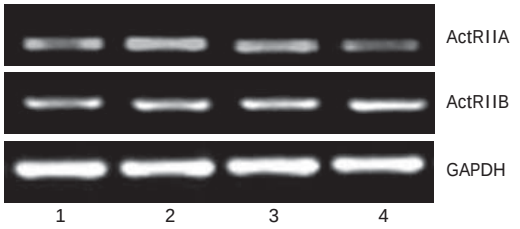

Figure 3 The mRNA expressions of ActRIIA and ActRIIB in ARIP2-overexpressed Hepal-6 cells. Lane 1 and 2: Hepal-6 cells were transfected with empty vector pcDNA3 (0.3 μg); lane 3: pcDNA3-ARIP2 (0.1 μg) + pcDNA3 (0.2 μg); lane 4: pcDNA3-ARIP2 (0.3 μg).

transfected into Hepal-6 cells and the effects of ARIP2 overexpression on ActRIIA and ActRIIB expression were observed in Hepal-6 cells. In this study, we found that ARIP2 overexpression could obviously suppress the expression of ActRIIA mRNA in Hepal-6 cells induced by activin A in dose-dependent manner, but has no effect on ActRIIB (Figure 3). These findings indicated that ARIP2 might down-regulated the expression of ActRIIA to suppress activin signal transduction in hepatocytes.

ARIP2 overexpression suppressed type Ⅳ collagen expression in Hepal-6 cells

The previous studies showed that activin A could induce liver fibrosis and stimulate excess secretion of ECM components, for example, collagen and fibronectin[3,16]. As an inhibitor of activin signal transduction, ARIP2 maybe influence the collagen production in hepatocytes induced by activin A. In this study, activin A could obviously stimulate the expression of type Ⅳ collagen mRNA in Hepal-6 cells. Whereas, after transfecting pcDNA3-ARIP2 into Hepa1-6 cells for 24 h, the ARIP2 overexpression could significantly suppress the expressions of type Ⅳ collagen mRNA induced by activin A in dose-dependent manner (Figure 4). To further determine the type Ⅳ collagen protein expression, the mature type Ⅳ collagen protein levels in Hepa1-6 cells were examined by flow cytometry. The results showed that ARIP2 overexpression could remarkably inhibit the expression levels of type Ⅳ collagen proteins in Hepa1-6 cells induced by activin A (the percent of positive fluorescence cells, 2% *vs* 16%) (Figure 5), which results were the same with that of type Ⅳ collagen mRNA by RT-PCR. These findings suggested that high level expression of ARIP2 might influence the expression of ECM components in hepatocytes and

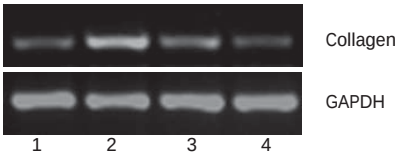

Figure 4 ARIP2-overexpression suppressed the expressions of collagen Ⅳ mRNA in Hepal-6 cells. lane 1 and 2: Hepal-6 cells were transfected with empty vector pcDNA3 (0.3 μg); lane 3: pcDNA3-ARIP2 (0.1 μg) + pcDNA3 (0.2 μg); lane 4: d pcDNA3-ARIP2 (0.3 μg). The cells were incubated in the absence (lane 1) or the presence of activin A (5 ng/mL) (lane 2, 3 and 4) for 24 h.

down-regulate the development of liver fibrosis induced by activin.

DISCUSSION

ARIPs have obvious expression and distribution diversity, which are key factors to the histological specificity of activin action[6-8]. It has been demonstrated that both ARIP1 and ARIP2 are inhibitors of activin signal transduction. However, ARIP1 mainly distributed in nerve tissues, ARIP2 widely existed in tissues. The high expression of ARIP2 mRNA could be detected in liver tissue by Northern blot[7]. In order to investigate the kinetic changes of ARIP2 expression, we used activin A and endotoxin LPS to stimulate Hepal-6 cells, and then examined the expression of ARIP2 mRNA. The results showed that the expression levels of ARIP2 mRNA were not changed obviously after being treated by activin in the early stage, but up-regulated depending on time 12 to 24 h after treatment (Figure 1). Stimulated by LPS, ARIP2 expression is also up-regulated evidently 12 h after treatment. In the present study, we further examined the effects of signal transduction activators PMA, A23187 and forskolin on the expression of ARIP2 in Hepal-6 cells (Figure 2). These data indicated that activin A, LPS, PMA and forskolin could promote the expression of ARIP2 mRNA in Hepal-6 cell, whereas calcium ion vector-A23187 could inhibit the expression of ARIP2 mRNA. These findings suggested that ARIP2 expression could be influenced by various factors and might participate in the regulation of signal transduction in the late stage in Hepal-6 cells.

Activin receptors are the members of serine/threonine kinase receptors[9,10]. Activin binds to receptor type Ⅱ to form a complex primarily. The complex interacts with the receptor type I and makes it phosphorylated, then activates endocellular Smad2/3 protein binding to receptor type I . Finally, it transduces signal into nucleus mediated by Smad4. Therefore, ActRIIs are the crucial receptors of activin signal transduction. In this study, we found that both ActR IIA and ActR IIB could be expressed in heapl-6 cells. To investigate the biological activities of ARIP2 expression in Hepal-6 cells, we transfected Hepal-6 cells with pcDNA3-ARIP2 and observed the effect of ARIP2 overexpression on ActRIIA and ActRIIB expressions in Hepal-6 cells. The results showed that ARIP2 overexpression could obviously suppress the expression of ActRIIA mRNA in Hepal-6 cells induced by activin A, but had no effect on ActRIIB (Figure 3). All the above data indicated that ARIP2 could down-regulated the expression of ActRIIA and participate in the process of negative feedback regulation of activin signal in hepatocytes.

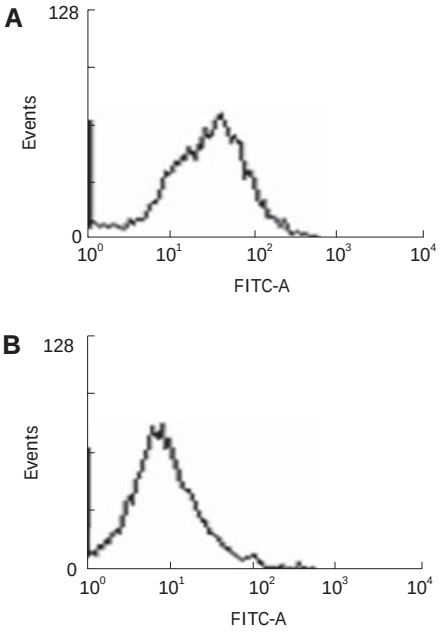

Figure 5 Flow cytometry analysis of collagen Ⅳ protein expressions in Hepal-6 cells. **A**: Hepal-6 cells were transfected with 0.3 μg pcDNA3; **B**: with 0.3 μg pcDNA3-ARIP2. 16% or 2% expressed the percent of positive fluorescence cells in **A** or **B**.

Activin not only play an important role in regulating secretion of hormone, but also serves as autocrine and paracrine factors to regulate the differentiation, proliferation, apoptosis of cells and embryonic development[17-21]. The latest studies have reported that as an important regulator, activin also has effects on inducing liver fibrosis, suppressing hepatocyte growth and so on[21-26]. It has been demonstrated that activin was produced by hepatocyte and hepatic stellate cell (HSC) and could promote HSC activation and stimulate excess production of ECM components, for example, collagen and fibronectin[3,16,25]. It has been reported that activin A could be expressed positively in fibrotic hepatocytes, and it also could take actions by autocrine[3,26]. In this study, we found that activin A could stimulate the expression of type Ⅳ collagen mRNA, whereas, the ARIP2 overexpression could remarkably suppress the mRNA expression of type Ⅳ collagen in Hepal-6 cells induced by activin A (Figure 4) and decrease the protein expression levels of type Ⅳ collagen. As a kind of collagen composed ECM, type Ⅳ collagen could co-deposited in Diss with fibronectin in the early stage of hepatic injury and take part in the formation of liver fibrosis. These findings suggested that ARIP2 overexpression might influence the synthesis of ECM in hepatocyte and negatively regulate the formation and development of liver fibrosis induced by activin A.

In conclusion, ARIP2 can be up-expressed in Hepal-6 cells by inducement with various factors and may participate in the regulation of signal transduction in the late stage. We may release or restraint liver diseases induced

by activin and achieve the goal of treatment if ARIP2 expression can be elevated in hepatocytes to inhibit the effects of activin.

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COMMENTS

Background

Activin A is involved in hepatic fibrosis formation. However, the mechanism of fibrotic process is not well understood. In this study, effects of anti-fibrosis by ARIP2 are investigated in mouse Hepal-6 cells.

Research frontiers

ARIP2 is a regulator of activin signaling pathway, but studies about its regulation in production of component of extracellular matrix (ECM)s are not reported.

Innovations and breakthroughs

Since ARIP2 has only been recently discovered, the mode of expression regulation and function of it have not been well characterized. We designed this experiment to investigate ARIP2 expression and its effects on the expression of collagen type Ⅳ by using Hepal-6 cells.

Applications

No ideal drug is available so far for the therapy of hepatic fibrosis. ARIP2 may be play an important role in regulation of development of liver fibrosis induced by activin.

Terminology

Activin receptor-interacting protein2 (ARIP2) can specifically interact with activin type Ⅱ receptor (ActRⅡ) and down-regulate intracellular signal transduction induced by activin.

Peer review

The topic is of interest for that up to now no antifibrotic therapy is available in patients with hepatic fibrosis. The negative effect of ARIP2 on production of component of extracellular matrix described in this paper shows that ARIP2 might be a potential treatment option.

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RAPID COMMUNICATION

Effects of large dose of dexamethasone on inflammatory mediators and pancreatic cell apoptosis of rats with severe acute pancreatitis

Xi-Ping Zhang, Li Chen, Qi-Fang Hu, Hua Tian, Ru-Jun Xu, Zhi-Wei Wang, Ke-Yi Wang, Qi-Hui Cheng, Wei Yan, Yun Li, Qing-Yu Li, Qing He, Fei Wang

Xi-Ping Zhang, Department of General Surgery, Hangzhou First People's Hospital, Hangzhou 310006, Zhejiang Province, China
Li Chen, Hua Tian, Department of General Surgery, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China
Qi-Fang Hu, Zhejiang Traditional Chinese Medicine University, Hangzhou 310053, Zhejiang Province, China
Ru-Jun Xu, Department of Pathology, Hangzhou First People's Hospital, Hangzhou 310006, Zhejiang Province, China
Zhi-Wei Wang, Ke-Yi Wang, Central Lab, Hangzhou First People's Hospital, Hangzhou 310006, Zhejiang Province, China
Qi-Hui Cheng, Department of Gynaecology and Obstetrics, Hangzhou First People's Hospital, Hangzhou 310006, Zhejiang Province, China
Wei Yan, Yun Li, Qing-Yu Li, Qing He, Fei Wang, Manufacturing Laboratory, Hangzhou First People's Hospital, Hangzhou 310006, Zhejiang Province, China
Supported by Grants for Traditional Chinese Medicine Science of Zhejiang Province, and Medical Science and Technology of Zhejiang Province and Hangzhou
Correspondence to: Xi-Ping Zhang MD, Department of General Surgery, Hangzhou First People's Hospital, 261 Huansha Road, Hangzhou 310006, Zhejiang Province, China. zxp99688@vip.163.com
Telephone: +86-571-87065701 Fax: +86-571-87914773
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Abstract

AIM: To investigate the influence of high dose of dexamethasone on inflammatory mediators and apoptosis of rats with severe acute pancreatitis (SAP).

METHODS: SAP rats were randomly assigned to the model group and treatment group while the normal rats were assigned to the sham operation group. The mortality, ascite volumes, ascites/body weight ratio and pancreas pathological changes of all rats were observed at 3, 6 and 12 h after operation. Their contents of amylase and endotoxin in plasma and contents of tumor necrosis factor (TNF-α), phospholipase A_2 (PLA_2) and IL-6 in serum were also determined. The microarray sections of their pancreatic tissues were prepared, terminal transferase dUTP nick end labeling (TUNEL) staining was performed and apoptotic indexes were calculated.

RESULTS: There was no marked difference between treatment group and model group in survival. The contents of amylase and endotoxin in plasma and contents of TNF-α, PLA_2 and IL-6 in serum, ascite volumes, ascites/body weight ratio and pancreas pathological scores were all lower in treatment group than in model group to different extents at different time points [$P < 0.05$, 58.3 (26.4) ng/L *vs* 77.535 (42.157) ng/L in TNF-α content, 8.00 (2.00) points *vs* 9.00 (2.00) points in pathological score of pancreas respectively; $P < 0.01$, 0.042 (0.018) EU/mL *vs* 0.056 (0.0195) EU/mL in endotoxin content, 7791 (1863) U/L *vs* 9195 (1298) U/L in plasma amylase content, 1.53 (0.79) *vs* 2.38 (1.10) in ascites/body weight ratio, 8.00 (1.00) points *vs* 11.00 (1.50) points in pathological score of pancreas; $P < 0.001$, 3.36 (1.56) ng/L *vs* 5.65 (1.08) ng/L in IL-6 content, 4.50 (2.00) *vs* 7.20 (2.00), 4.20 (1.60) *vs* 6.40 (2.30), 3.40 (2.70) *vs* 7.90 (1.70) in ascite volumes, respectively]. The apoptotic indexes of pancreas head and pancreas tail were all higher in treatment group than in model group at 6 h [$P < 0.01$, 0.00 (2.00)% *vs* 0.00 (0.00)%, 0.20 (1.80) *vs* 0.00 (0.00) in apoptosis indexes, respectively].

CONCLUSION: The mechanism of dexamethasone treatment in acute pancreatitis is related to its inhibition of inflammatory mediator generation and induction of pancreatic acinar cell apoptosis.

Key words: Severe acute pancreatitis; Apoptosis; Inflammatory mediators; Dexamethasone; Tissue microarrays

Zhang XP, Chen L, Hu QF, Tian H, Xu RJ, Wang ZW, Wang KY, Cheng QH, Yan W, Li Y, Li QY, He Q, Wang F. Effects of large dose of dexamethasone on inflammatory mediators and pancreatic cell apoptosis of rats with severe acute pancreatitis. *World J Gastroenterol* 2007; 13(41): 5506-5511

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INTRODUCTION

The pathogenesis of severe acute pancreatitis (SAP) is closely related to the factors such as activation of pancreatin, release of inflammatory mediators, microcirculation

disturbance and apoptosis. The sound therapeutic effects of large dose of dexamethasone on SAP have been demonstrated. In this experiment, the mechanism of large dose of dexamethasone in SAP was discussed and the changes of inflammatory mediator content and pancreatic acinar cell apoptosis after dexamethasone treatment for SAP rats were observed. The tissue microarray has also been applied to the pathohistological examination of pancreatitis to improve the study efficiency.

MATERIALS AND METHODS

Materials

Clean grade healthy male Sprague-Dawley (SD) rats with body weight of 250-300 g were purchased from the Experimental Animal Center of Medical School, Zhejiang University. Sodium taurocholate and pentobarbital were purchased from USA Sigma Company, and dexamethasone injection from Zhejiang Xinchang Pharmaceutical Company, China. The full automatic biochemical analyzer was used to determine the plasma amylase level (U/L). Plasma endotoxin tachypleus amebocyte lysate kit was purchased from Shanghai Yihua Medical Science and Technology Corporation (Institute of Medical Analysis, Shanghai, China), the calculation unit is EU/mL. The TNF-α ELISA kit was purchased from Jingmei Bioengineering Corporation, the calculation unit is pg/mL (ng/L). The serum secretory phospholipase A_2 enzyme assay ELA kit (PLA_2) was purchased from R&D System Institute and the calculation unit is U/mL. The above determinations were all operated according to the instructions of the kits.

Animal grouping and rat SAP model preparation

Ninety clean grade healthy male SD rats were prepared into SAP models by the improved Aho's method and randomly divided into the model group (45 rats) and treatment group (45 rats). Another 45 were assigned into the sham operation group. The above groups were then randomly divided into the 3, 6 and 12 h group with 15 rats in each. The treatment group was injected with dexamethasone via vena caudalis, 0.5 mg/100 g body weight, 15 min after successful preparation of SAP model. In the sham operation group, pancreas and duodenum were turned over before the abdomen was closed. The sham operation group and model group were injected with the saline of the same volume *via* vena caudalis 15 min after the operation[1]. SAP model was established according to the reference[1].

Observation indexes

The rat mortality was determined at 3, 6 and 12 h after operation and the survival rate was calculated at different time points.

After the rats were anesthetized by sodium pentobarbital and killed in batches, the pancreas samples were collected. Fix them according to the related requirements, observe the pathological changes of pancreas after HE staining and compare the pathological scores among groups. The standard of pancreas pathological score was in accordance with reference[2]. The content of amylase, endotoxin in plasma, and TNF-α, IL-6 and PLA_2 in serum of all groups were determined at different time points.

Table 1 Comparison of ascite volumes [M (Q_R)]

Group	3 h	6 h	12 h
Sham operation group	0.50 (0.00)	0.70 (0.50)	0.60 (0.30)
Model group	7.20 (2.00)	6.40 (2.30)	7.90 (1.70)
Dexamethasone treated group	4.50 (2.00)[b]	4.20 (1.60)[b]	3.40 (2.70)[b]

[b]$P < 0.001$, dexamethasone treated group *vs* model group.

Apoptotic indexes

The tissue microarray was applied to prepare the tissue microarray sections of pancreas, which were stained by DNA, and terminal transferase dUTP nick end labeling (TUNEL). The observation of pancreatic cells and calculation of apoptotic indexes were carried out respectively.

Statistical analysis

The statistical analysis was conducted with the SPSS11.5 software. The Kruskal-Wallis test or variance analysis (only applied to PLA_2) was performed for the comparison among the three groups. The Bonfferoni test was also applied to the comparison. There are statistical significances when $P < 0.05$.

RESULTS

Survival

The mortality of model group was 0% (0/15), 0% (0/15) and 13.33% (2/15) at 3, 6 and 12 h, respectively. The sham operation group and dexamethasone treated group survived at all time points while there was no marked difference between the model group and dexamethasone treated group ($P > 0.05$)[1].

Comparison of ascite volumes

The model group and treated group had significantly higher ascite columes than sham operation group ($P < 0.001$), while the treatment group had significantly lower ascite volume than the model group ($P < 0.001$) (Table 1).

Comparison of ascites/body weight ratio

The model group and treatment group had significantly higher ascites/body weight ratio than sham operation group ($P < 0.001$), while the treatment group had significantly lower ratio than the model group at 3 h ($P < 0.01$), and the treatment group had significantly lower ratio than the model group at 6 and 12 h ($P < 0.001$) (Table 2).

Comparison of plasma amylase content

The plasma amylase content in model group and dexamethasone treated group was significantly higher than in the sham operation group at all time points ($P < 0.001$). There was no marked difference between the dexamethasone treated group and model group at 3

Table 2 Comparison of ascites/body weight ratio [M (Q_R)]

Group	3 h	6 h	12 h
Sham operation group	0.20 (0.04)	0.30 (0 .30)	0.22 (0.10)
Model group	2.38 (1.10)	2.58 (0.70)	2.54 (0.71)
Dexamethasone treated group	1.53 (0.79)[b]	1.40 (0.63)[d]	1.36 (0.74)[d]

[b]$P < 0.01$, [d]$P < 0.001$, dexamethasone treated group *vs* model group.

and 6 h ($P > 0.05$). The plasma amylase content in the dexamethasone treated group was significantly less than in the model group at 12 h ($P < 0.01$) (Table 3).

Comparison of plasma endotoxin content

The plasma endotoxin content in the model group and dexamethasone treated group was significantly higher than in the sham operation group at all time points ($P < 0.001$). No marked difference was found between the dexamethasone treated group and model group at 3 h ($P > 0.05$). The content in dexamethasone treated group was significantly less than in the model group at 6 and 12 h ($P < 0.01$) (Table 3).

Comparison of serum TNF-α content

The model group and dexamethasone treated group had significantly higher serum TNF-α content than the sham operation group at all time points ($P < 0.001$). No marked difference was noticed between the dexamethasone treated group and model group at 3 h ($P > 0.05$). The dexamethasone treated group had significantly less serum TNF-α content than the model group at 6 and 12 h ($P < 0.05$)[1] (Table 3).

Comparison of serum IL-6 content

The serum IL-6 contents of model group and treatment group were significantly higher than those of sham operation group ($P < 0.001$); the content of treatment group was significantly lower than that of model group at 3 h ($P < 0.01$); and the contents of treatment group were significantly lower than those of model group at 6 and 12 h ($P < 0.001$) (Table 3).

Comparison of serum PLA_2 content

The model group and dexamethasone treated group significantly higher serum PLA_2 content than the sham operation group at all time points ($P < 0.001$). The content in the dexamethasone treated group was significantly less than in the model group ($P < 0.001$) (Table 4).

Pathological score of pancreatic tissue

HE staining was performed and the pathohistological score standard was referred to the improved Schmidt score. Two chief pathologists used the blind method for scoring[2].

Gross pathological changes of pancreas. (1) Sham operation group: No apparent abnormality of pancreas and peripancreatic epiploon at all time points. (2) Model group: The gross pathological change of pancreas tail was more apparent than that of pancreas head. The severity of overall pathological change increased with time after modeling. At 3 h, a small amount of hemorrhagic ascites was observed by naked eyes with relatively apparent changes of pancreas hyperemia and edema, hemorrhage and necrosis; at 6 and 12 h, hemorrhagic ascites increased more apparently with edema, hemorrhage and necrosis, and more saponified spots could be seen on peripancreatic epiploon and peritoneum. (3) Treatment group: At 3 h, the degree of pancreas hyperemia and edema, hemorrhage and necrosis was milder than that of model group with decrease of ascitic fluid; at 6 and 12 h, the pancreatic hemorrhage and necrosis area and degree were milder than those of model group with apparent decrease of ascitic fluid.

The pancreas pathological changes under light microscope. (1) Sham operation group: Mild interstitial edema occurred in a few cases, and neutrophil infiltration was occasional. No acinar cell, fat necrosis and hemorrhage were observed. (2) Model group: The pathological change severity increased with time after modeling. At 3 h, pancreas interstitial hyperemia, edema, a small amount of inflammatory cell infiltration, focal necrosis and interstitial hemorrhage occurred, among which some were lamellar hemorrhage and necrosis. At 6 h, interstitial edema, hemorrhage, inflammatory cell infiltration, focal and lamellar hemorrhage and necrosis occurred. At 12 h, large area of hemorrhage and necrosis, lobule outline damage and a large amount of inflammatory cell infiltration were found. (3) Treatment group: The pathological change scope and degree of most cases were milder than those of model group at corresponding time points. Only a few had lamellar hemorrhage and necrosis, but the scope of hemorrhage and necrosis decreased and inflammatory cell infiltration apparently alleviated.

Comparison of pathological score of pancreas

Both model group and dexamethasone group had significantly higher pathological score of pancreas than the sham operation group at different time points ($P < 0.01$) while that in dexamethasone group was significantly less than in the model group at 3 and 6 h ($P < 0.05$), and it was also significantly less in the dexamethasone group than in the model group at 12 h ($P < 0.01$) (Table 5).

Comparison of apoptosis indexes

The apoptosis index of pancreas head and tail at 3 and 12 h was not significantly different among all groups ($P > 0.05$). No marked difference was found between the model group and sham operation group at different time points ($P > 0.05$). At 6 h, the apoptosis index of pancreas in the treatment group was significantly higher in the model group and sham operation group ($P < 0.01$) (Table 6, Figure 1A and B).

Correlation analysis

There was a positive correlation between amylase and PLA_2 of model group at 3 h ($P < 0.05$); the TNF-α content of treatment group was positively correlated with PLA_2 at 6 h ($P < 0.05$). There was a positive correlation between pancreas pathological score and TNF-α ($P < 0.05$).

Table 3 Comparison of different indexes level in blood [M (Q_R)]

Index	Sham operation group			Model group			Dexamethasone treated group		
	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h
Amylase	2038	2117	1725	7423	8149	9195	6739	7839	7791[b]
(U/L)	(346)	(324)	(434)	(2275)	(1540)	(1298)	(2310)	(2258)	(1863)
Endotoxin	0.015	0.015	0.016	0.035	0.055	0.056	0.03	0.040[b]	0.042[b]
(EU/mL)	(0.007)	(0.007)	(0.005)	(0.017)	(0.025)	(0.0195)	(0.014)	(0.012)	(0.018)
TNF-α	3.3	4.9	3.7	46.125	77.535	67.301	38.4	58.3[a]	38.7[a]
(ng/L)	(3.6)	(2.6)	(2.3)	(37.954)	(42.157)	(32.1315)	(26.6)	(26.4)	(28.5)
IL-6	1.75	1.75	1.48	4.87	6.65	5.65	3.31[b]	3.17[b]	3.36[b]
(ng/L)	(0.65)	(1.04)	(0.57)	(1.38)	(1.45)	(1.08)	(1.38)	(1.28)	(1.56)

[a]$P < 0.05$, [b]$P < 0.01$, dexamethasone treated group *vs* model group.

Table 4 Comparison of serum PLA_2 content (mean ± SD, U/mL)

Group	3 h	6 h	12 h
Sham operation group	18.70 ± 4.40	16.70 ± 3.83	18.52 ± 11.32
Model group	103.70 ± 20.82	119.85 ± 17.74	121.29 ± 17.00
Dexamethasone treated group	53.96 ± 15.4[b]	67.75 ± 27.95[b]	65.27 ± 26.21[b]

[b]P < 0.001, dexamethasone treated group *vs* model group.

Table 5 Comparison of pathological score of pancreas [M (Q_R)]

Group	3 h	6 h	12 h
Sham operation group	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Model group	8.00 (2.00)	9.00 (2.00)	11.00 (1.50)
Dexamethasone treated group	7.00 (2.00)[a]	8.00 (2.00)[a]	8.00 (1.00)[b]

[a]$P < 0.05$, [b]$P < 0.01$, dexamethasone treated group *vs* model group.

Table 6 Comparison of apoptosis index of the head and tail of pancreas [M (Q_R)]

Group (*t*/h)	Pancreas head			Pancreas tail		
	3 h	6 h	12 h	3 h	6 h	12 h
Sham operationgroup	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Model group	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Dexamethasone treated group	0.00 (0.00)	0.00 (2.00)[b]	0.00 (0.00)	0.00 (0.00)	0.20 (1.80)[b]	0.00 (0.00)

[b]$P < 0.01$, dexamethasone treated group *vs* model group.

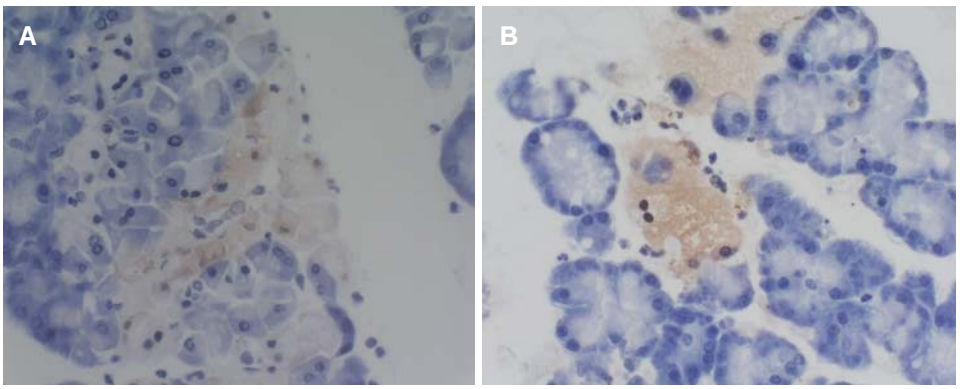

Figure 1 **A**: Dexamethasone treated group-6 h (Apoptosis of pancreatic acinar cell); **B**: Dexamethasone treated group-6 h (Apoptosis of pancreatic acinar cell). (TUNEL × 400).

DISCUSSION

Under normal circumstances, the inflammatory mediators are at low level of dynamic balance to maintain the stability of the internal environment. Excessive inflammatory reaction plays a vital role in SAP pathogenesis[3-6]. In this experiment, the influence of dexamethasone on inflammatory mediators in treatment of SAP rats was studied and its relationship with the apoptosis of pancreatic acinar cells was discussed.

TNF-α can increase the local tissue damage and capillary permeability, eventually aggravating pancreatic necrosis, which is important in AP[7,8]. Norman *et al*[7] found in SAP rats a positive correlation between the TNF-α concentrations in pancreatic tissue and plasma and the level of pancreatic injury and inflammation, which is consistent to the fact that in this experiment, the pancreas pathological score was positively correlated with TNF-α at 12 h in model group ($P < 0.05$). It was found in this experiment that the serum TNF-α contents were lower

in treatment group than in model group at 6 and 12 h ($P < 0.05$), demonstrating that dexamethasone plays a certain role in inhibiting serum TNF-α content. Since PLA_2 plays an important role in SAP onset[8-10], PLA_2 antagonist can significantly improve the pathological injury of pancreas of the animal model with pancreatic injury[11,12]. In this study, the serum PLA_2 content was lower in treatment group than in model group ($P < 0.001$), demonstrating a significant inhibiting effect of dexamethasone on PLA_2 or its generation. IL-6, mainly generated by monocyte/macrophage, T cell, B cell, *etc*, participates in many acute body reactions such as burn, sepsis and major operation. The positive correlation between serum IL-6 level and AP severity has been proved by many studies[13]. And the histological score of pancreas can be significantly improved by lowering IL-6. The serum IL-6 contents were all lower in treatment group than in model group to different extents ($P < 0.01$ or $P < 0.001$), demonstrating that dexamethasone can inhibit serum IL-6 content.

In recent years, it was found that both inflammatory mediators and pancreatic acinar cell apoptosis are related to AP[14,15]. Apoptosis participates in AP onset[16]. The apoptosis of pancreatic acinar cell might be a reaction beneficial to the body after the occurrence of pancreatitis[17,18]. Both necrosis and apoptosis are death modes of injured cells[19]. However, substantially different from necrosis, apoptosis will not release the harmful substance in lysosome or cause intense inflammatory reaction[20]. Necrosis prevails in SAP. The illness can be alleviated by apoptosis induction and aggravated by apoptosis inhibition[14]. In this experiment, according to the result of TUNEL staining, the apoptotic index was higher in treatment group than in model group ($P < 0.01$), and the pathological score was lower in treatment group than in model group ($P < 0.05$) at 6 h, demonstrating that dexamethasone can promote the apoptosis of pancreatic cells and protect pancreatic tissue.

The effect of glucocorticoid (represented by dexamethasone) on AP/SAP has been an issue in dispute. In 1952, Stephensen *et al*[21] for the first time reported the effect of glucocorticoid in AP treatment. Many empirical studies show glucocorticoid can improve the survival of AP animals[22,23] Its mechanisms mainly are: inhibiting the generation of inflammatory mediators and (or) inhibiting the effects of inflammatory mediators, enhancing body stress, improving microcirculation, alleviating endotoxemia, cleaning free radicals, inhibiting nitric oxide (NO) and expression of NF-κB, *etc*[24-26]. In terms of administration and dose, Dong *et al*[27,28] found a large dose of dexamethasone was obviously superior to the small dose dexamethasone in therapeutic effect and early use of dexamethasone was superior to dexamethasone of the same dose 5 h later. We used large doses of dexamethasone and achieved relatively sound therapeutic effects, obviously alleviating pathological changes of pancreas.

This empirical study used the improved Aho's method[29] to prepare SAP model, the rat survival of the dexamethasone treated group was significantly higher than that of the model group, but there was no marked difference between the two groups ($P > 0.05$). However, no matter gross or under light microscope, the treatment group has milder pancreatic tissue cell inflammatory pathological changes, less ascitic fluid and hemorrhage, and lower necrosis scope than the model group at all time points.

The contents of amylase and endotoxin in plasma and TNF-α, PLA_2 and IL-6 in serum were all lower in dexamethasone treated group than in model group. The apoptotic index was higher in treatment group than in model group while the inflammation, hemorrhage and necrosis of pancreas were all milder in treatment group than in model group, indicating that dexamethasone can improve the pancreatic injury of SAP rats by directly inducing pancreatic cell apoptosis, or indirectly inducing apoptosis through inhibition of excessive rise of TNF-α, IL-6, PLA_2, *etc*. In this experiment, no relation has been found between inflammatory mediators and pancreas apoptotic index. However, it has been found in many studies that the inflammatory mediators released by injured cells in AP can influence apoptosis. The role of inflammatory mediators that indirectly regulate apoptotic gene is significant and non-neglectable during its participation in apoptosis. It is worth mentioning that there are various but one influential factors act together to result in apoptosis during AP, presenting a network relation structure[30].

We used the tissue microarray section maker (Beecher Instruments, USA) to drill a hole 2.0 mm in diameter on recipient block and combined TUNEL staining method to examine the apoptotic index. The results indicate that the tissue chip 2.0 mm in diameter can achieve reliable experimental result, which is representative, time, energy and reagent saving, and convenient for control.

COMMENTS

Background

The severe acute pancreatitis (SAP) is one of the common acute abdomens in clinical practice. The pathogenesis of SAP is closely related to factors such as activation of pancreatin, release of inflammatory mediators, microcirculation disturbance and apoptosis. The recent studies prove the apoptosis could be a beneficial reaction to AP. The apoptosis of acinar cell in pancreas inducing injury can alleviate the inflammatory reaction. In this experiment, the mechanism of dexamethasone treatment in SAP was discussed and the changes of inflammatory mediator content and pancreatic acinar cell apoptosis were observed.

Research frontiers

To discuss the influence of dexamethasone on inflammatory mediators and apoptosis of rats with SAP, the authors established the rat SAP models and combined the tissue microarrays to observe the influence of dexamethasone on apoptosis of acinar cell in pancreas, providing a new theoretical basis for dexamethasone treatment of SAP and application of tissue microarrays in pancreatitis pathological examinations.

Innovations and breakthroughs

The tissue microarray has been applied to the pathohistological examination of pancreatitis to improve the study efficiency.

Applications

The sound therapeutic effects of a large dose of dexamethasone on SAP have been demonstrated. It is of some value to apply tissue microarrays to pathological examination and analysis of non-tumor diseases like pancreatitis.

Terminology

The apoptosis is a kind of self-protecting fashion namely the body starts the autogene program under certain pathological and physiological conditions and removes the irreparable cells, which is substantially different from necrosis. Tissue microarray (TMA), or tissue chip, is a method of harvesting small disks (diameter 0.6-2.0 mm) of tissue from a range of standard histologic sections and placing in an array on a recipient paraffin block by which hundreds of cases can be analyzed simultaneously. This technique allows maximization of tissue resources by analysis of small-core biopsies of blocks, rather than complete sections.

Peer review

Through animal studies, the authors investigated the influence of dexamethasone on inflammatory mediators and apoptosis of rats with severe acute pancreatitis. They concluded that the mechanism of dexamethasone treatment in acute pancreatitis is related to its inhibition of inflammatory mediator generation and induction of pancreatic acinar cell apoptosis.

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CASE REPORT

Histological changes at an endosonography-guided biliary drainage site: A case report

Naotaka Fujita, Yutaka Noda, Go Kobayashi, Kei Ito, Takashi Obana, Jun Horaguchi, Osamu Takasawa, Kazunari Nakahara

Naotaka Fujita, Yutaka Noda, Go Kobayashi, Kei Ito, Takashi Obana, Jun Horaguchi, Osamu Takasawa, Kazunari Nakahara, Department of Gastroenterology, Sendai City Medical Center, Sendai, Japan
Correspondence to: Naotaka Fujita, Department of Gastroenterology, Sendai City Medical Center, 5-22-1, Tsurugaya, Miyagino-ku, Sendai, Miyagi 983-0824, Japan. docfujita@gmail.com
Telephone: +81-22-2521111 Fax: +81-22-2529431
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Abstract

Endosonography-guided biliary drainage (ESBD) is a new method enabling internal drainage of an obstructed bile duct. However, the histological conditions associated with fistula development *via* the duodenum to the bile duct have not been reported. We performed ESBD 14 d preoperatively in a patient with an ampullary carcinoma and histologically confirmed changes in and around the fistula. The female patient developed no complications relevant to ESBD. Levels of serum bilirubin and hepatobiliary enzymes declined quickly, and pancreatoduodenectomy was carried out uneventfully. The resected specimen was sliced and stained with hematoxylin-eosin. Histological evaluation of the puncture site in the duodenum and bile-duct wall, and the sinus tract revealed no hematoma, bile leakage, or abscess in or around the sinus tract. Little sign of granulation, fibrosis, and inflammatory cell infiltration was observed. Although further large-scale confirmatory studies are needed, the findings here may encourage more active use of ESBD as a substitute for percutaneous transhepatic drainage in cases with failed/difficult endoscopic biliary stenting.

Key words: Endosonography; Endoscopic ultrasound-guided fine needle aspiration; Endoscopic biliary drainage; Biliary stenting; Endoscopic retrograde cholang iopancreatography; Obstructive jaundice; Biliary stricture

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INTRODUCTION

The role of endosonography (ES) in digestive diseases is gradually expanding from diagnostic to therapeutic applications. In the mid 1990s, the feasibility of ES-guided cholangiopancreatography was first reported by Harada *et al*[1] (pancreatography) and Wiersema *et al*[2] (cholangiography). Several reports on the application of this technique for therapeutic purposes, such as ES as a guide for biliary drainage, have been published.

We have recently applied ES-guided biliary drainage (ESBD) for preoperative decompression of the biliary tree in a patient with cancer of the papilla of Vater. The results of histological evaluation of and around the sinus tract are reported herein.

CASE REPORT

A 76-year-old Japanese woman was admitted to our department, complaining of jaundice and itching. Laboratory data on admission showed the following abnormalities: serum total bilirubin, 11.7 mg/dL; glutamic oxaloacetic transaminase (GOT), 389 IU/L; glutamic pyruvic transaminase (GPT), 285 IU/L; and alkaline phosphatase (ALP), 1487 IU/L. Transabdominal ultrasonography and abdominal computed tomography revealed a mass in the ampullary region, along with dilatation of the bile and pancreatic ducts (Figure 1). To resolve her complaints, endoscopic retrograde cholangiopancreatography (ERCP) with biliary stenting was attempted. However, cannulation of the bile duct was unsuccessful because stenosis of the descending portion of the duodenum, and the presence of a tumor at the papilla of Vater that bled easily when contacted, made it impossible to manipulate the endoscope to identify the orifice. After obtaining written informed consent, ESBD was undertaken. Following visualization of the extrahepatic bile duct with a curved linear array echoendoscope (GF-UC240P; Olympus, Tokyo, Japan), the dilated bile duct was punctured *via* the upper part of the descending portion of the duodenum with a 19G needle (Olympus) (Figure 2A). Following removal of the core needle, white bile was aspirated. A small amount of contrast agent was then injected *via* the sheath catheter into the bile duct, to guide stent placement and to confirm the absence of bile leakage or extravasation. A guidewire 0.889 mm in diameter (Jagwire; Boston Scientific, Natik, MA, USA) was introduced into the sheath catheter and inserted into the intrahepatic bile duct. Subsequent to

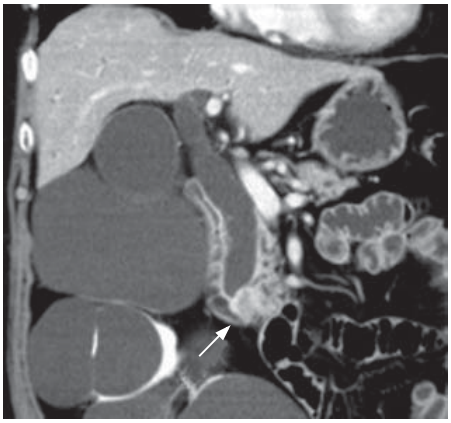

Figure 1 CT reveals a mass in the ampullary region (arrow) with a dilated extrahepatic bile duct. The patient also had multiple renal cysts.

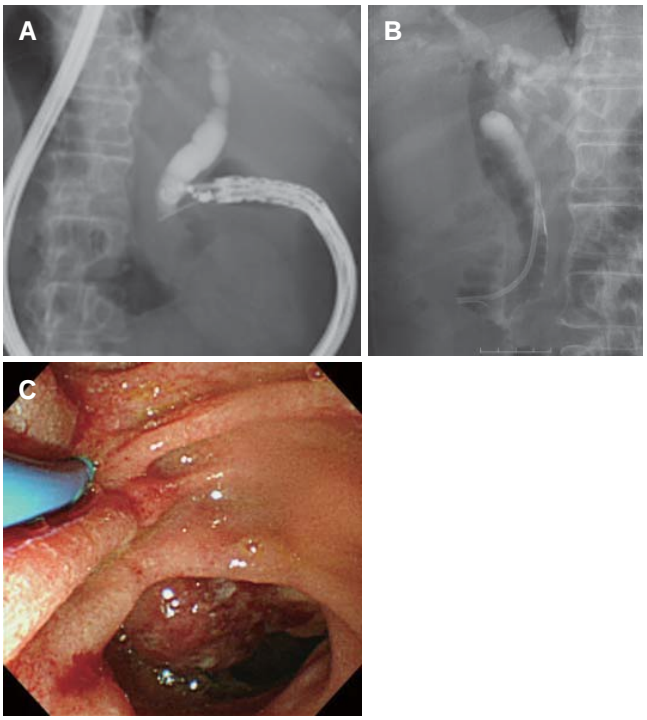

Figure 2 A, B: Puncture of the bile duct via the duodenum under endosonographic guidance, followed by deployment of a plastic stent. **C:** Endoscopic view after stent placement.

removing the sheath catheter and leaving the guidewire *in situ*, we dilated the puncture tract with a dilator catheter 7F in diameter, and placed a 7F plastic stent (Figure 2B and C). The patient developed no symptoms related to the procedure and her initial complaints also soon disappeared. One week after the procedure, levels of serum total bilirubin, GOT, GPT, and ALP had declined to 3.2 mg/dL, 33 IU/L, 47 IU/L, and 780 IU/L, respectively. She underwent pancreaticoduodenectomy 14 d after ESBD. Macroscopically, the sites of the puncture in the bile duct and duodenum were clear without infection, hemorrhage or hematoma (Figure 3).Histological examination revealed mild inflammatory cell infiltrate adjacent to the sinus tract in the duodenal and bile duct walls, without hemorrhage (Figure 4). A fistula was formed

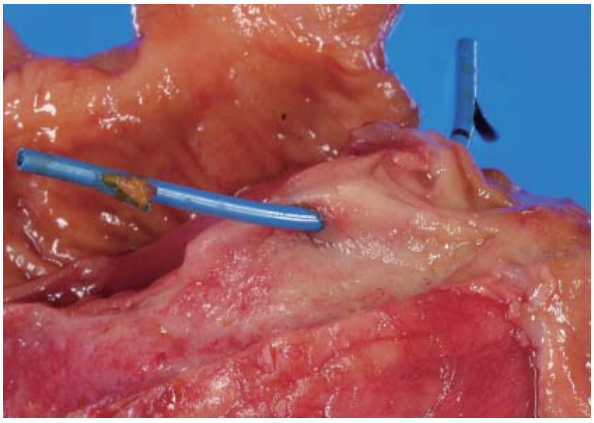

Figure 3 Fresh resected specimen. No hematoma or abscess is seen at the site of the puncture in the bile duct.

along the tract of the puncture without significant reactive changes. No evidence of severe inflammation, such as bile peritonitis, was found on the extraluminal side of either the duodenum or the bile duct.

DISCUSSION

The role of ES in the management of gastrointestinal diseases has evolved from imaging of the gut wall and organs adjacent to the alimentary tract, to its use as a guide for tissue sampling with a fine-needle, as well as in therapeutic applications such as injection of agents into tumors and drainage of pancreatic pseudocysts. Endosonographic approaches to an inaccessible bile duct and pancreatic duct by ERCP were first reported in the mid 1990s. In 1996, Wiersema *et al*[2] reported the feasibility of ES-guided cholangiopancreatography. Subsequently, several case series have been published on the feasibility and effectiveness of ESBD, the first being by Giovannini *et al*[3] in 2001, in a pancreatic cancer patient with a history of failed cannulation of the bile duct, even after precutting, who underwent preoperative chemoradiotherapy. They succeeded in the deployment of a 10F stent in a two-step procedure by changing endoscopes. In 2003, Burmester *et al*[4] reported three cases of successful stent placement among four attempts at ESBD. They achieved biliary drainage by a one-step technique, a modification of the Seifert technique[9] for drainage of pancreatic pseudocysts. They approached the bile duct via the duodenum, stomach and even the jejunum. Mallery *et al*[5] performed endoscopic ultrasound-guided rendezvous drainage of the bile duct and pancreatic duct after unsuccessful ERCP. They succeeded in stent placement following antegrade traverse of the stenosis with a guidewire in three (two biliary and one pancreatic) of six cases. Puspok *et al*[6] applied this technique to cases of bile duct stones with difficult cannulation of the bile duct, and reported excellent results. Kahaleh *et al*[7] recently published their experience of ESBD in 23 patients. They carried out puncture of the bile duct *via* the stomach, duodenum and jejunum, and concluded that intrahepatic access to the biliary system appears safer than the extrahepatic approach.

As described here, acceptable success rates and inci-

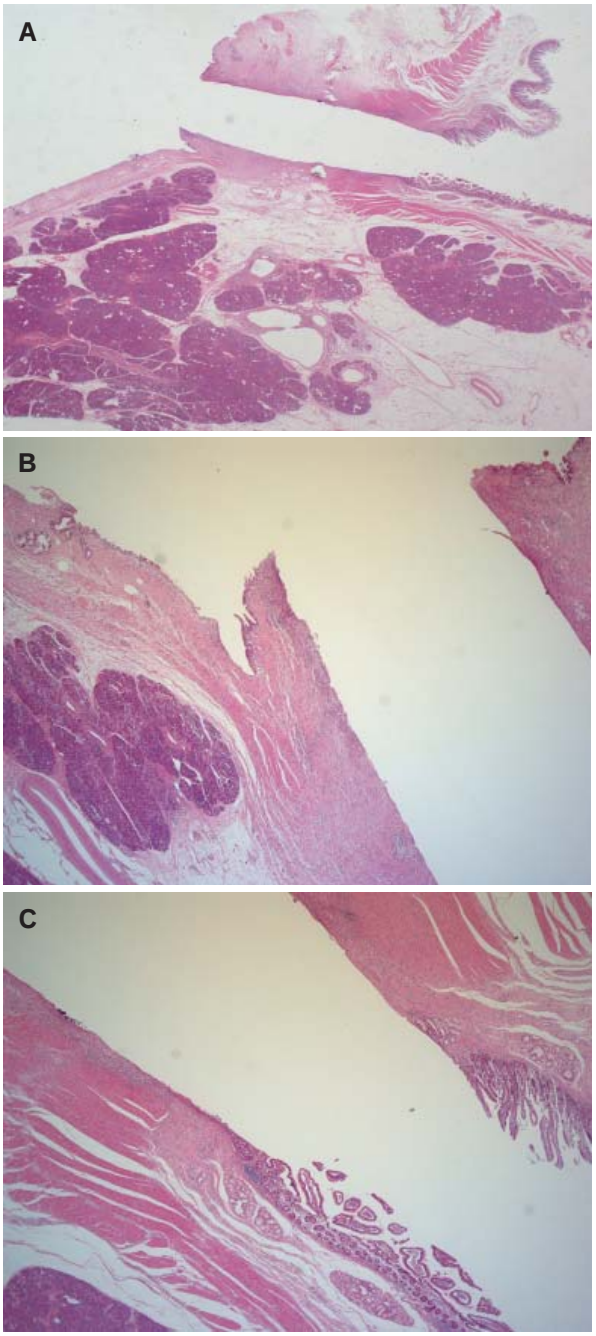

Figure 4 Microscopic views of the sinus tract. Mild inflammatory cell infiltrate adjacent to the sinus tract in the duodenal wall and the bile duct wall is seen, without hemorrhage or abscess formation. A fistula is formed along the tract of the puncture but without significant reactive changes. **A:** Low-power view of the sinus tract (× 1.25); **B:** End of the sinus tract on the bile duct side (× 5); **C:** End of the sinus tract on the duodenal side (× 5).

dence of complications were reported for puncture and stent placement under sonographic guidance. However, there have been no reports of the influence of this technique on the gut wall, bile duct, or intervening tissues between them. This is believed to be the first report describing the preoperative performance of ESBD and the histological condition of the sinus tract established by this method. The duodenum, bile duct and sinus tract showed no adverse histological changes. These results are attributable to the use of endosonography as a guide. This technique has a low potential risk of major bleeding as color Doppler evaluation is utilized for determining the route of the puncture. Injury to adjacent organs is also minimized due to clear visualization of the structures in the area of interest, with the use of high-frequency ultrasound. Some authors[4,6,8] have applied a fistulotome with electrocautery for puncture. However, it seems theoretically safer to apply a simple puncture needle to avoid damage to the tissues in and around the pathway of the puncture.

The present study elucidated histological changes at and around the sinus tract, following ESBD. The results that no severe inflammation or hemorrhage occurred should encourage the wider use of this technique, although further large-scale studies will be needed.

In general, the method of choice for biliary obstruction is endoscopic biliary stenting. Percutaneous transhepatic cholangio-drainage (PTCD) is considered a substitute. However, PTCD can result in pain after placement of the drainage tube, and can restrict activities of daily living. In contrast, ESBD is a safe and effective method for biliary drainage and does not cause pain or restriction of daily living, as is the case with endoscopic biliary stenting. It should therefore replace PTCD in a large proportion of those patients with an obstructed biliary tree and with difficult cannulation of the bile duct, duodenal stenosis, or deformity of the papilla of Vater caused by cancer, which hinders detection of the orifice, regardless of the likelihood of successful PTCD.

As is the case with plastic stents in endoscopic biliary stenting, the diameter of the stent available in ESBD is restricted by the diameter of the working channel of the endoscope employed. The endoscope we used had a 2.8-mm diameter working channel, allowing the use of only 7F stents, or smaller.

Dilation of the sinus tract can be achieved with a dilator balloon, following insertion of the guidewire into the bile duct *via* the lumen of the placed stent and removal of the sent alone. Therefore, once access to the bile duct has been established, it is possible to place a stent with a larger caliber, even a metallic stent, without difficulty in either a one- or two-step procedure. In the long term, it is inevitable that plastic stents will become occluded. Deployment of a metallic stent will prolong patency, as shown in endoscopic transpapillary biliary stenting[10]. Covered metallic stents are deemed to be advantageous in avoiding bile leakage.

Further development of accessory devices specialized for ESBD will help expand its indications.

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CASE REPORT

Synchronous isolated splenic metastasis from colon carcinoma and concomitant splenic abscess: A case report and review of the literature

Adolfo Pisanu, Alberto Ravarino, Riccardo Nieddu, Alessandro Uccheddu

Adolfo Pisanu, Alberto Ravarino, Riccardo Nieddu, Alessandro Uccheddu, Department of Surgery; Department of Pathology, University of Cagliari, Clinica Chirurgica, Cagliari, Italy
Supported by grant from the University of Cagliari, Italy
Correspondence to: Dr. Adolfo Pisanu, Clinica Chirurgica, Università degli Studi di Cagliari, Ospedale San Giovanni di Dio, Via Ospedale 46, Cagliari 09124, Italy. adolfo.pisanu2@tiscali.it
Telephone: +39-70-6092420 Fax: +39-70-6092417
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Abstract

This study aimed to describe a case in which an isolated splenic metastasis was synchronous with the colonic primary and a concomitant splenic abscess was associated. A wide review of the literature was also performed. A 54-year-old woman with abdominal pain and fever was admitted to our department. Abdominal CT revealed two low-density areas in the spleen and wall-thickening of the left colonic flexure, which was indistinguishable from the spleen parenchyma. The patient underwent emergency celiotomy, with the presumptive diagnosis of obstructing colon carcinoma of the splenic flexure, and concomitant splenic abscess. Subtotal colectomy and splenectomy were performed. Pathological findings were consistent with mucinous colonic carcinoma, synchronous isolated splenic metastasis and concomitant splenic abscess. This paper is also a review of the existing literature on the association between colorectal cancer and splenic metastasis. Only 41 cases of isolated splenic metastasis from colon carcinoma have been reported in the literature. This report is the third described case of synchronous isolated splenic metastasis from colon carcinoma. Only one case with concomitant splenic abscess has been previously reported. When obstructing left-sided colorectal cancer is suspected, careful CT examination can allow early diagnosis of splenic involvement by the tumor. The literature review suggests that there might be a significant improvement in survival following splenectomy for a metachronous isolated splenic metastasis from colon carcinoma. Prognosis for synchronous splenic metastasis seems to be related to the advanced stage of the disease. Nevertheless, no definitive conclusions can be drawn because of the small number of cases.

INTRODUCTION

Primary and metastatic tumors of the spleen are described as unusual[1], excluding secondary involvement by lymphoma[2]. Since metastatic carcinoma involving the spleen is usually a manifestation of widely disseminated disease, isolated splenic metastasis from colorectal carcinoma is not a common occurrence[1,3]. Its rareness has been hypothetically explained by several characteristics of the spleen, such as anatomical, histological and immunological features[4]. Most cases are asymptomatic and the diagnosis is usually made by imaging studies during the diagnostic work up for colon cancer[5]. However, a few patients become exceptionally symptomatic following spontaneous rupture of the spleen, or the presence of an associated splenic abscess[6,7].

We report the case of a synchronous isolated splenic metastasis from colonic carcinoma, with a concomitant splenic abscess, and we also review all cases of isolated splenic metastasis from colorectal cancer reported in the literature. To the best of our knowledge, only one case of splenic metastasis from colonic carcinoma associated with concomitant splenic abscess has been reported in the literature[7], which is an extremely rare clinical entity.

CASE REPORT

In June 2006, a 54-year-old Caucasian woman was referred to our emergency department because of abdominal pain associated with intermittent fever over 40℃, shaking and chills. She also complained of general fatigue and loss of appetite. Otherwise, her previous medical history was unremarkable. On clinical examination, the patient was pale and shocked. Blood pressure and pulse rate were 90/60 mmHg and 98/min, respectively. The abdomen was distended, with tenderness in the left hypochondrium. There

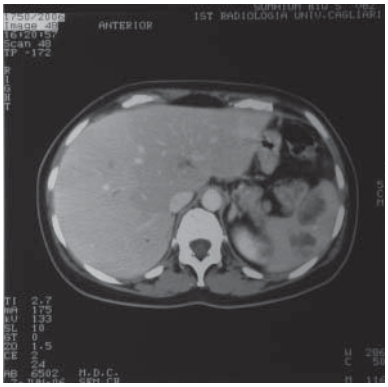

Figure 1 Two low density areas in the spleen (Axial CT-scan).

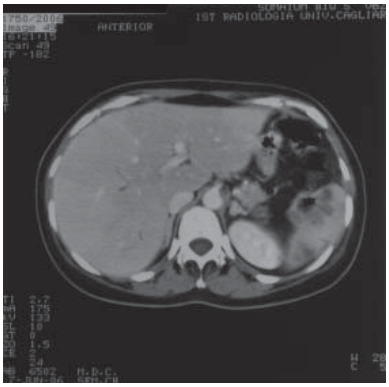

Figure 2 Wall thickening of the left flexure of the colon indistinguishable from the spleen parenchyma (Axial CT-scan).

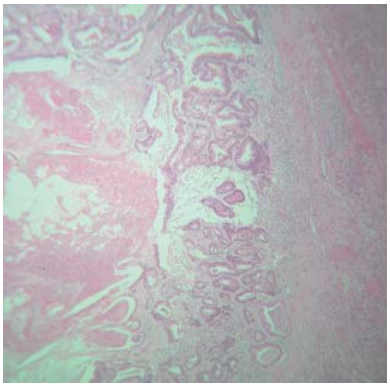

Figure 3 Splenic tumor showing glandular pattern consistent with metastasis from colonic mucinous carcinoma (HE, x 40).

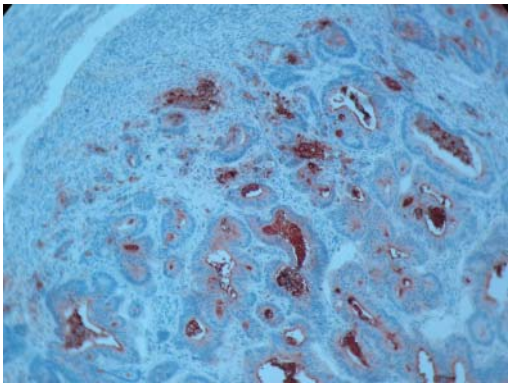

Figure 4 CEA along the luminal border of tumor cells infiltrating splenic pulp (anti-CEA monoclonal antibody staining, x 100).

was neither hepatosplenomegaly nor lymphadenopathy. Sepsis was identified both by a clear clinical picture which showed high fever and hemodynamic instability, and by positive blood culture which grew *Escherichia coli* and *Bacteroides fragilis*. Laboratory data on hospital admission were as follows: white blood cell count, 148 × 10^9/L; red blood cell count, 280 × 10^{12}/μL; hemoglobin, 67 g/L; thrombocyte count 383× 10^9/L; fibrinogen, 5.17 g/L; albumin 23 ug/L; and carcinoembryonic antigen (CEA), 31.1 ug/L (normal range < 2.50 ug/L).

Chest X-ray revealed a left pleural effusion. Abdominal plain radiography showed intestinal obstruction with typical air fluid levels in the bowel. Abdominal ultrasonography demonstrated a hypoechoic area with unclear margins in the lower pole of the spleen, with the features of a splenic abscess. Enhanced abdominal CT revealed two low-density areas in the spleen (Figure 1) and wall-thickening of the left colonic flexure, which was indistinguishable from the spleen parenchyma (Figure 2). Echocardiography was normal and detected no vegetations. The patient was treated with fluid, intensive antibiotics and blood transfusion as initial therapy, and then she underwent an emergency operation for a presumptive diagnosis of obstructing colonic carcinoma and septic shock from a concomitant splenic abscess. On explorative celiotomy, the splenic flexure of the colon presented a mass occluding the lumen, infiltrating the entire colonic wall, and invading the lower pole of the spleen. There were neither hepatic metastases nor peritoneal dissemination. Frozen section examination of the spleen was performed after splenectomy. Frozen section showed the presence of splenic metastasis from adenocarcinoma, with a concomitant splenic abscess. A subtotal colectomy with side-to-side ileo-sigmoid anastomosis was then performed. The spleen weighed 160 g and measured 12 cm × 7 cm × 4.5 cm. On gross examination, the tumor originated from the left colonic flexure and invaded the spleen, in which it formed a fistula and an abscess in the metastatic tissue. The splenic metastasis measured 4.5 cm at its largest diameter and had a central abscess with a cavity of 2 cm. Histopathological findings were consistent with mucinous adenocarcinoma of the colon, synchronous isolated splenic metastasis from the primary colonic tumor (Figure 3), and concomitant splenic abscess, without metastatic lymph-node involvement (T4N0M1). Immunohistochemistry of both colonic carcinoma and splenic metastasis was performed using anti-CEA monoclonal antibody (Clone Ⅱ-7, Dako Corporation, Carpinteria, CA, USA). Staining of CEA along the luminal border of tumor cells was demonstrated both in the colonic carcinoma and in the metastasis that infiltrated the splenic pulp (Figure 4). Left pleural effusion persisted for 10 d after the operation, and the patient was finally discharged on postoperative day 15.

After 1 mo, the CEA level dropped to 3.0 μg/L. The patient was treated with adjuvant chemotherapy. Six months after the operation, CEA level was 10.4 μg/L. Abdominal CT and positron emission tomography (PET) revealed a solitary liver metastasis of 2 cm, which was surgically removed. Exploration of the abdominal cavity revealed no further evidence of neoplastic disease. Afterward, the patient was once more subjected to adjuvant chemotherapy.

Table 1 Literature review of isolated splenic metastasis from colorectal carcinoma

Year	Author	Journal	Site of primary tumor	Synchronous metastasis	Metachronous metastasis	Concomitant splenic abscess
1969	Dunbar[10]	Mayo Clinic Proc	Rectum		1	
1982	Waller[25]	Clin Nucl Med	Sigmoid colon		1	
1986	Slavin[26]	Clin Nucl Med	Right colon		1	
1992	Capizzi[27]	South Med J	Rectum		1	
1993	Thomas[28]	Eur J Surg Oncol	Left colon		1	
1997	Mainprize[3]	Br J Surg	Splenic flexure		1	
1997	Indudhara[13]	South Med J	Sigmoid colon		1	
1999	Achuthan[6]	Ann R Coll Surg Engl	Rectum		1	
1999	Weathers[24]	Dis Colon Rectum[1]	Sigmoid colon		1	
1999	Vadalà[29]	Minerva Chir	Left colon		1	
2000	Kim[4]	J Korean Med Sci	Right colon		1	
2000	Lee[30]	Am Surg	Not specified		1	
2001	Place[1]	Am Surg	Sigmoid colon		1	
2001	Avesani[11]	Am J Clin Oncol	Left colon	1		
2001	Paramelle[7]	J Radiol	Left colon	1		1
2001	Okuyama[20]	Jpn J Clin Oncol	Sigmoid colon		1	
2001	Quoted in Okuyama[20]	Jpn J Clin Oncol[2]	Left colon		11	
			Left + right colon		1	
			Right colon		7	
			Rectum		1	
2003	Genna[17]	Minerva Chir	Left colon		1	
2004	Cavallaro[12]	J Exp Clin Cancer Res	Sigmoid colon		1	
2004	Pizzirusso[31]	Acta Chir Belg	Left colon		1	
2006	Cabanas[16]	Tumori	Sigmoid colon		1	
2006	Gencosmanoglu[5]	World J Surg Oncol	Sigmoid colon + splenic flexure		1	
2007	Pisanu	Present report	Splenic flexure	1		1
			Total	3	39	2

[1]This metastasis occurred 3 mo after colonic operation; [2]From the review of the Japanese literature.

DISCUSSION

Approximately 20% of colorectal carcinomas are metastatic at their clinical presentation[8]. Metastases to other sites in the absence of liver, lung or axial skeleton involvement are very rare[1,9]. Similar to the results of an autopsy study by Berge, microscopic splenic metastases were found in 7%-34% of cancer patients[2]. The same author reported the incidence of splenic micrometastases arising from colorectal carcinoma to be as high as 2% in 1019 colorectal tumors, but all of these involved other organs as well[2]. In 1969 Dunbar *et al*[10] published the first case report of isolated splenic metastasis from colorectal carcinoma and to date, only 41 such cases have been reported in the literature (Table 1). All but two of previously described cases of solitary splenic metastases from colon carcinoma had a metachronous metastasis (Table 1). We have described the third reported case in which an isolated splenic lesion was synchronous with colonic carcinoma[7,11]. The particularly interesting aspect of our case was also related to the simultaneous presence of a splenic abscess, because the only other reported case with similar clinical and pathological features is that by Paramelle *et al*[7].

The rareness of splenic metastasis arising from colonic carcinoma suggests the existence of some mechanism that prohibits tumor cell proliferation in the spleen. Anatomical and immunological characteristics may be reasons for the rarity of isolated splenic metastasis[4]. From an anatomical perspective, the sharp angle of the splenic artery with the celiac axis and rhythmic contraction by the sinusoidal splenic architecture are limiting factors for metastasis[4,12]. According to Indudhara *et al*[13], neoplastic cells can reach the splenic vein and parenchyma by retrograde diffusion through the inferior mesenteric vein. The spleen parenchyma contains no afferent lymphatic vessels, but they are present in the capsular, subcapsular and trabecular regions[12]. Tumor cells might also reach the spleen *via* the lymphatic system, which explains the typical subcapsular localization of isolated splenic metastases[12]. As the spleen is the second largest organ of the reticuloendothelial system, immune surveillance appears to potently inhibit tumor cell proliferation[14]. Moreover, experimental studies have shown that the growth rate of adenocarcinoma cells injected into the spleen is significantly lower than that of the same cells injected into the liver[15].

Histopathological findings in our case were consistent with mucinous adenocarcinoma of the colon, as in three other cases of isolated splenic metastasis[3,16,17]. Mucinous gastrointestinal malignancies are thought to cause perforation and infiltration of the full thickness of the bowel wall, which lead to extensive invasion of the pericolic fat[3,16]. A new mechanism has been proposed in the case of contiguous splenic metastasis from mucinous colonic tumors. Cabanas *et al*[16] have suggested that mucus-producing epithelial cells become trapped within the trabecula of the splenic capsule, in congenital clefts of the spleen, or in microfissures caused by trauma. The resistance of the splenic capsule or fibrous tissue surrounding the spleen causes the mucinous tumor to expand into the soft splenic parenchyma, rather than

the peritoneal cavity[16]. Following the more aggressive behavior of mucinous colonic tumors, this mechanism may have explained the presence of the synchronous splenic metastasis in our case. Furthermore, since CEA appears as an immunosuppressant, and acts as an adhesion molecule between tumor cells and visceral macrophages, colonic tumor cells with positive CEA staining should display more aggressive behavior[4,18]. In regard to these biological functions, CEA expression might be associated with the occurrence of isolated splenic metastasis[4] (Figure 4).

The diagnosis of isolated splenic metastasis is generally made by imaging studies during the diagnostic work up for colonic cancer[5]. Only a few patients with splenic metastasis become symptomatic because of the presence of an associated splenic abscess[8] or spontaneous rupture of the spleen[6,19], as in our case. Okuyama *et al*[20] have pointed out that only six of 28 reported patients were symptomatic at the time of diagnosis. Our patient became symptomatic as a result of abdominal occlusion and sepsis originating from the splenic abscess, in which *E. coli* and *B. fragilis* grew, as in most cases of colonic abscess associated with colonic cancer[21]. The association between splenic abscess and colonic cancer is a very rare clinical entity[22], as is isolated splenic metastasis. Only a few cases have been reported in the literature, and sometimes the splenic abscess resulted from a direct fistula of descending colon carcinoma, without spleen metastasis[23]. The most frequent complication of splenic abscess is its rupture into the peritoneal cavity, and untreated splenic abscesses have a high mortality rate[23].

Most previously described patients with solitary splenic metastasis from colon carcinoma had a disease-free survival of 3-144 mo after the primary tumor[11,17,24]. Long-term survival after splenectomy in patients with isolated metachronous splenic metastasis from colon carcinoma varied from 0.5 to 7 years[11,20,24]. As a result, prognosis of isolated splenic metastasis after splenectomy appears to be rather optimistic, despite the fact that splenic metastasis is one form of distant metastasis[20]. In our case of synchronous metastasis, intensive follow-up revealed a solitary liver metastasis 6 mo after operation, without further evidence of neoplastic disease in the abdominal cavity. However, the disease-free interval after splenectomy in the case of synchronous splenic metastasis reported by Avesani *et al*[11] was 10 mo, and the patients died of diffuse carcinomatosis after 1 year.

The spleen is considered unfavorable to the development of metastases but the reason for this is not clearly understood. An isolated splenic metastasis from colon carcinoma is a rare clinical finding. On the basis of the present case, when an obstructing left-sided colorectal cancer is suspected in emergency setting, careful examination of the abdominal CT-scan can allow early diagnosis of a splenic involvement by the tumor. Clinicians must pay close attention to the spleen for the early diagnosis of isolated splenic metastasis when routinely evaluating abdominal CT-scan and abdominal ultrasonography following curative resection of primary colorectal cancer. Splenectomy is necessary in the presence of isolated metastases from colon carcinoma both synchronous and methacronous. The occurrence of a splenic abscess makes emergency splenectomy mandatory as the most frequent complication is its rupture into the peritoneal cavity.

Splenectomy followed by chemotherapy seems to be the preferred treatment of isolated splenic metastases from colorectal carcinoma. There are few data available about recurrence after splenectomy for metastases of this type. Literature review suggests that there might be a significant improvement of long-term survival following splenectomy for methacronous splenic metastasis arising from colon carcinoma. Prognosis for synchronous splenic metastasis seems to be related to the advanced stage of the disease. Nevertheless, following the small number of cases reported in the literature, no definitive conclusions can be drawn.

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CASE REPORT

Benign retroperitoneal schwannoma presenting as colitis: A case report

Gary Fass, Didier Hossey, Michel Nyst, Dirk Smets, Esmail Najar Saligheh, Ruth Duttmann, Kathleen Claes, Pierre Mendes da Costa

Gary Fass, Didier Hossey, Michel Nyst, Dirk Smets, Pierre Mendes da Costa, Department of Digestive, Laparoscopic and Thoracic Surgery, Brugmann University Hospital, Brussels, Belgium
Esmail Najar Saligheh, Department of Radiology, Brugmann University Hospital, Brussels, Belgium
Ruth Duttmann, Department of Pathology, Brugmann University Hospital, Brussels, Belgium
Kathleen Claes, Laboratory of Molecular Genetics, Centre for Medical Genetics, Ghent University Hospital, Ghent, Belgium
Correspondence to: Gary Fass, Department of Digestive, Laparoscopic and Thoracic Surgery, Brugmann University Hospital, Place Van Gehuchten 4 Brussels 1020, Belgium. garyfass@hotmail.com
Telephone: +32-477-180354 Fax: +32-3-4483714
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Abstract

We report a case of a patient presenting with clinical, radiological and endoscopic features of colitis due to a compressive left para-aortic mass. Total open surgical excision was performed, which resulted in complete resolution of colitis. Histopathology and immunohistochemistry revealed benign retroperitoneal schwannoma. These neural sheath tumors rarely occur in the retroperitoneum. They are usually asymptomatic but as they enlarge they may compress adjacent structures, which leads to a wide spectrum of non-specific symptoms, including lumbar pain, headache, secondary hypertension, abdominal pain and renal colicky pain. CT and MR findings show characteristic features, but none are specific. Schwannoma can be isolated sporadic lesions, or associated with schwannomatosis or neurofibromatosis type Ⅱ (NF2). Although they vary in biological and clinical behavior, their presence is, in nearly every case, due to alterations or absence of the *NF2* gene, which is involved in the growth regulation of Schwann cells. Both conditions were excluded by thorough mutation analysis. Diagnosis is based on histopathological examination and immunohistochemistry. Total excision is therapeutic and has a good prognosis. Schwannomatosis and NF2 should be excluded through clinical diagnostic criteria. Genetic testing of NF2 is probably not justified in the presence of a solitary retroperitoneal schwannoma.

Key words: Colitis; Neurofibromatosis; Retroperitoneum; Schwannoma

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INTRODUCTION

Schwannomas or neurilemmomas are encapsulated tumors arising from the neural sheath of peripheral nerves. They are usually present in the head and neck or in the upper extremities, but may appear in the posterior mediastinum and more rarely in the retroperitoneum. The latter are often found incidentally or may present with vague, non-specific symptoms if the tumor is large enough to compress surrounding structures.

We report a case of a benign retroperitoneal schwannoma with an unusual clinical presentation, its radiological, histopathological and genetic features, and its subsequent management.

CASE REPORT

A 45-year-old woman was admitted to our emergency department for severe colicky abdominal pain with nausea and vomiting that started about 15 min after her last meal. She had no relevant medical history but mentioned having had back pain for the last 2 mo, which she thought was due to her increasing workload and stress at work. She denied having any abdominal problems until that day. On clinical examination, the patient was afebrile, pale and uncomfortable. Blood pressure and heart rate were normal. Abdominal examination showed a diffusely tender abdomen, more pronounced in the left lower quadrant. She had no guarding or rebound. Deep palpation of the abdomen revealed a non-tender, non-pulsatile mass left of the umbilicus. Rectal examination was unremarkable, except for the presence of a little blood. Within the first hour after admission, the patient suffered one episode of diarrhea stained with a moderate amount of fresh blood. Laboratory tests showed an increased white blood cell count

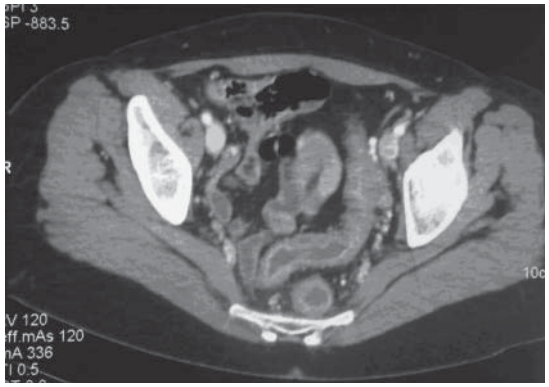

Figure 1 Contrast-enhanced CT scan of the abdomen showing a diffuse infiltration around the rectum and the sigmoid colon, and thickening of their walls.

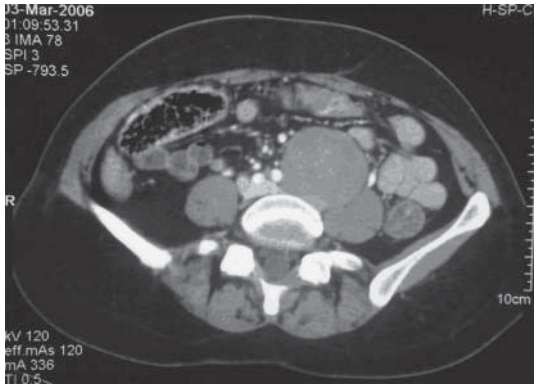

Figure 2 Well-demarcated, homogeneous mass measuring 60×50 mm in close proximity to the left iliac artery, lumbar vertebrae and psoas muscle, on contrast-enhanced CT scanning.

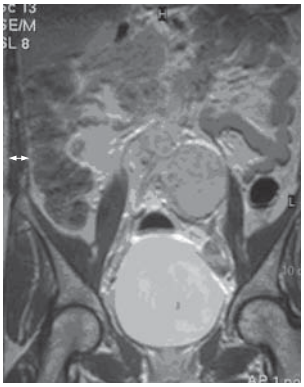

Figure 3 Coronal T_1-weighted MR image using gadolinium, showing a solid mass with the same features as seen with CT scanning.

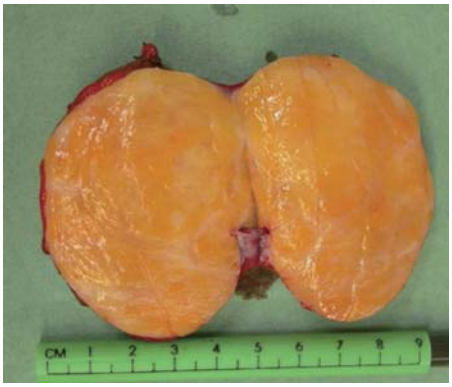

Figure 4 Perioperative examination of the mass revealed a solid, greyish, ovoid tumor with a smooth capsule and a homogeneous yellow core.

(WBC) of 18 000 cells/mm³ and C-reactive protein (CRP) of 1.1 mg/dL. Other values were within the normal range. At this point our differential diagnosis included diverticulitis and a contrast-enhanced computed tomodensitometry (CT) of the abdomen was performed. It revealed a diffuse infiltration around the rectum and the sigmoid colon, and a thickening of their walls (Figure 1). No diverticula were found. It also showed a well-demarcated, homogeneous, left para-aortic mass lying between the lumbar vertebrae and the left psoas muscle, which measured 60 × 50 mm (Figure 2). With few specific findings, the scan was considered inconclusive.

She was hospitalized and received isotonic fluid resuscitation, and intravenous broad-spectrum antibiotics were commenced. Symptoms improved rapidly and disappeared almost completely 3 d after the initial complaints. During her hospital stay, several examinations were performed. First, the patient underwent colonoscopy to exclude inflammatory bowel disease (IBD). It revealed a diffusely edematous, slightly erythematous rectum and sigmoid colon, but no erosive or ulcerative lesions. Histopathological examination of biopsies taken during colonoscopy showed diffuse edema and signs of chronic inflammation, characterized by the presence of mostly lymphocytes and polynuclear granulocytes. Granulomas were absent. A few cryptic abscesses and zones of erosion were also found. The pathologist's diagnosis was chronic colitis, more pronounced in the sigmoid colon than the left colon, and excluded IBD. In laboratory tests, WBC had fallen to a normal value at 9780 cells/mm³ after a rise to 12 000. CRP level followed the same course to settle at 1.7 mg/dL, after it had risen to 10.8. Tumor markers CEA and CA 19.9 were within the normal range. Magnetic resonance imaging showed the same features as seen with CT scanning (Figure 3).

Since we believed that the mass caused the patient's signs and symptoms, we opted for open surgical excision. We approached the retroperitoneal space through a median laparotomy. During exploration and meticulous dissection of the mass, we noticed that it was in close proximity to the aorta and left iliac artery and vein, and seemed to arise from the left para-aortic sympathetic chain. It also adhered partially to the left psoas muscle and to the anterior aspect of the lumbar vertebrae. Complete excision was performed. Perioperative examination of the mass revealed a solid, greyish, spherical tumor with a smooth capsule and a heterogeneous core (Figure 4).

The postoperative course was uneventful. We noticed that the left leg was slightly warmer and dryer than the right, which suggested that we had performed a left lumbar sympathectomy, thus in favor of the excision of a tumor of neural origin. Microscopic histopathological examination revealed strings of spindle cells surrounded by a collagenous stroma that was partially hyalinized and showed cystic degeneration in some regions (Figure 5). There was some nuclear atypia with very limited mitotic activity, but

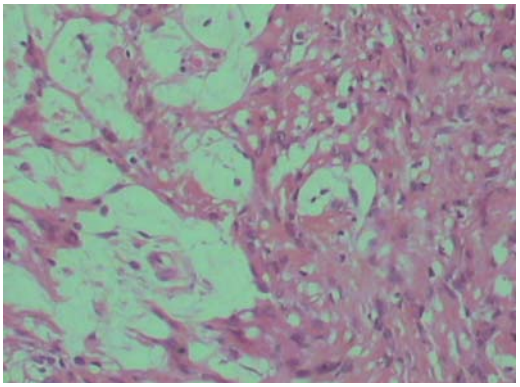

Figure 5 Antoni A area on the right (well-organised spindle cells in a palisade pattern) and Antoni B area (less cellular, loose pleomorphic cells) on the left (HE, × 200).

no signs of malignancy. The tumor tested intensely positive for S-100 protein, which confirmed the diagnosis of benign schwannoma. The integrity of the capsule was also noted, which confirmed total excision of the mass. The patient was discharged from the hospital on the fifth postoperative day.

Six weeks after surgery, she was still symptom free and signs of sympathectomy persisted. A CT scan and colonoscopy were performed to evaluate the evolution of colitis, which had completely resolved. Further investigations were performed to exclude neurofibromatosis type Ⅱ (NF2) or schwannomatosis. Thorough physical examination was performed on the patient but no superficial tumors were found. Family history for schwannoma or meningioma was negative. MRI did not show any tumor of the central nervous system. Furthermore, the presence of a germline *NF2* mutation was excluded by thorough mutation analysis on DNA extracted from the patient' s lymphocytes. We performed direct sequencing of all coding exons of the *NF2* gene. Deletion was excluded by multiplex ligation-dependent probe amplification (MLPA). We concluded that the schwannoma was most likely due to a somatic mutation in the *NF2* gene.

DISCUSSION

Schwannomas, or neurilemmomas, are tumors arising from the Schwann cells of peripheral nerves[1,2]. They are usually found in the head and neck or in the upper extremities. Only 1% is found in the retroperitoneum, which accounts for 0.5%-1.2% of all retroperioneal tumors[3,4]. They can be isolated as sporadic lesions or associated with schwannomatosis or NF2. Although they vary in biological and clinical behavior, their presence is in nearly every case due to alterations or absence of the *NF2* gene (located on chromosome 22q12), which codes for *merlin*, a tumor suppressor protein involved in the growth regulation of Schwann cells, but its exact mechanism has not yet been elucidated. Most schwannomas are benign but (although very rarely) malignant degeneration can occur, and is usually associated with NF2[5]. Patients with benign retroperitoneal schwannomas are predominantly in their second to fifth decade, and women are twice as often affected as men[4,6].

On gross appearance, schwannomas are well-demarcated, solid tumors with a smooth surface and have an ovoid or spherical shape[7]. Sometimes, secondary changes such as hemorrhage, cysts and calcification can be present[1]. They are usually solitary and slow-growing tumors[1]. The retroperitoneum is non-restrictive, so that benign tumors are often able to grow to a large size before causing symptoms. These are generally vague and non-specific[7], and range from lumbar pain and neurological symptoms in the lower extremities[8], to renal colicky pain, with or without hematuria, if it involves the urogenital tract[9]. Abdominal complaints can also occur but are mainly vague and poorly localized, with some digestive disturbances[3,10,11]. Our patient's presentation was peculiar, not only because of the abrupt onset of her symptoms, but also because she had colitis. We believe that the tumor was large enough to compromise venous return in the mesocolon, which led to stasis characterized by the infiltration and parietal thickening seen on CT, the edematous and erythematous aspect seen during colonoscopy, and the chronic inflammation with edema seen on histopathological examination. This idea has been strengthened by the fact that those signs disappeared completely after removal of the tumor.

Diagnosis is rarely made preoperatively. The mass seen on the CT scan showed characteristic features of benign schwannoma. It was a well-demarcated, spherical, solitary mass and in a paravertebral position[6]. Contrast enhancement homogeneity was seen because no gross cystic degeneration or calcification had yet occurred. Most authors agree that these features are not diagnostic. Other diagnoses such as paraganglioma, neurofibroma, ganglioneuroma, tumors of mesodermal origin and retroperitoneal malignancies, including malignant fibrous histiocytoma, lymphoma and liposarcoma, should be considered[12,13]. MRI was helpful because it has a better definition, multiplanar capabilities, and the possibility to differentiate the nature of the tumor, such as solid tissue, fibrous tissue, simple or atypical fluid, and blood[14]. It confirmed the presence of a solid, homogeneous mass, and showed its relation to adjacent structures in greater detail[6,15]. No invasive process was revealed and the margins were still regular, convincing us that the mass was benign in nature[16]. Definite diagnosis was made during histopathological examination and immunohistochemistry. Microscopically, the mass showed Antoni A (well-organised spindle cells in a palisade pattern) and B (less cellular, loose pleomorphic cells) areas[1,2], and tested intensely positive for S-100 protein, which is almost exclusively identified within benign nerve sheath tumors[17,18]. CT-guided fine needle aspiration biopsy can be helpful in determining the origin of a mass preoperatively, but is seldom accurate[7]. Since we believed the mass was causing colitis, open surgical excision was performed. Successful laparoscopic removal of retroperitoneal schwannomas has been reported[19], as well as with endoscopic minilaparotomy[20].

Recurrence is rare and probably due to incomplete excision[7,21]. Further investigations were performed to exclude schwannomatosis or NF2. Absence of other schwannomas and lack of family history of schwannoma theoretically excluded both conditions (see diagnostic criteria in Tables 1 and 2). The presence of a germline *NF2* mutation was excluded by a thorough genetic analysis. We performed direct

Table 1 Diagnostic criteria for NF2[22]

Definite NF2
1 Bilateral vestibular schwannomas or
2 Family history of NF2 (first-degree family relative) plus
   a Unilateral vestibular schwannoma at age < 30 yr, or
   b Any two of the following: meningioma, glioma, schwannoma or juvenile posterior subcapsular lenticular opacities/juvenile cortical cataract

Presumptive or probable NF2
1 Unilateral vestibular schwannoma at age < 30 yr plus at least one of the following: meningioma, glioma, schwannoma or juvenile posterior subcapsular lenticular opacities/juvenile cortical cataract
2 Multiple meningiomas (two or more) plus
   a Unilateral vestibular schwannoma at age < 30 yr, or
   b One of the following: glioma, schwannoma or juvenile posterior subcapsular lenticular opacities/juvenile cortical cataract

Table 2 Diagnostic criteria for schwannomatosis[23]

Definite schwannomatosis
1 Two or more pathologically proved schwannomas, plus
2 Lack of radiographic evidence of vestibular schwannoma at age > 18 yr

Presumptive or probable schwannomatosis
1 Two or more pathologically proved schwannomas without symptoms of eighth nerve dysfunction at age > 30 yr or
2 Two or more pathologically proved schwannomas in an anatomically limited distribution (single limb or segment of the spine), without symptoms of eighth nerve dysfunction, at any age

sequencing of all coding exons of the *NF2* gene. Deletion was excluded by MLPA. This mutation detection strategy, ideally performed on the original tumor specimen, allows the detection of a germline mutation in > 90% of NF2 patients with a family history of the disease, and in > 70% of sporadic cases. However, it is probably only justified in sporadic unilateral vestibular schwannoma in patients aged < 20 years, unless other features of NF2 are present[24].

In conclusion, benign retroperitoneal schwannomas are rare tumors arising from the neural sheath of peripheral nerves. They are usually incidental findings but may become symptomatic if sufficiently large. Symptoms are usually vague and non-specific and can mimic different diseases. CT and MR findings show characteristic features, but none are specific. Diagnosis is based on histopathological examination and immunohistochemistry. Total excision is therapeutic and has a good prognosis. Genetic testing for NF2 is probably not justified in the presence of a solitary retroperitoneal schwannoma.

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CASE REPORT

Gallstone spillage caused by spontaneously perforated hemorrhagic cholecystitis

Young Chul Kim, Mi-Suk Park, Yong Eun Chung, Joon Suk Lim, Myeong-Jin Kim, Ki Whang Kim

Young Chul Kim, Mi-Suk Park, Yong Eun Chung, Joon Suk Lim, Myeong-Jin Kim, Ki Whang Kim, Department of Diagnostic Radiology and Institute of Gastroenterology, Severance Hospital, Research Institute of Radiological Science, Yonsei University College of Medicine, Seoul, South Korea
Correspondence to: Mi-Suk Park, MD, Department of Diagnostic Radiology, Severance Hospital, Seodaemun-ku, Shinchon-dong 134, Seoul 120-752, South Korea. radpms@yumc.yonsei.ac.kr
Telephone: +82-2-22287400 Fax: +82-2-3933035
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Abstract

There are occasional incidences of gallstone spillage during laparoscopic cholecystectomy, and there have been frequent reports on such a topic in the literature. To the best of our knowledge, however, there have been no reports about spilled stones caused by spontaneously perforated hemorrhagic cholecystitis. Here, we report the radiologic findings of spilled stones caused by spontaneously perforated hemorrhagic cholecystitis in a 55-year-old man.

Key words: Gallbladder perforation; Gallstone spillage; Hemorrhagic cholecystitis

Kim YC, Park MS, Chung YE, Lim JS, Kim MJ, Kim KW. Gallstone spillage caused by spontaneously perforated hemorrhagic cholecystitis. *World J Gastroenterol* 2007; 13(41): 5525-5526

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INTRODUCTION

With the increased use of laparoscopic surgery, the spillage of gallstones during laparoscopic cholecystectomy has been reported in 6%-40% of cases[1,2]. To the best of our knowledge, however, there have been no reports about spilled stones caused by spontaneously perforated hemorrhagic cholecystitis. Here, we present ultrasonography (US) and computed tomography (CT) images of this rare condition.

CASE REPORT

A 55-year-old man complained of abrupt upper abdominal pain during hospitalization for a brain abscess. A complete blood count taken 12 h after the attack showed that the level of hemoglobin dropped to 8.8 g/L (from 13.3 g/L, 36 h before the attack). Other blood analysis revealed mild thrombocytopenia (platelet count $106 \times 10^3/\mu L$), and mild hyperbilirubinemia (total bilirubin concentration 1.7 mg/dL); however, the white blood cell count was normal ($9.27 \times 10^6/\mu L$).

Immediately after the attack, the patient underwent US, which demonstrated echogenic material in the gallbladder lumen (Figure 1A), with a positive sonographic Murphy's sign. US was discontinued because the patient complained of severe abdominal pain. Contrast-enhanced CT was performed and its images revealed high-density fluid, both inside and outside the gallbladder. One impacted cystic duct stone was seen, as were several calcified objects (which looked like stones), within the high-density (46-61 HU) fluid surrounding the gallbladder (Figure 1B and C). A defect in the wall or mucosal disruption of the gallbladder was also noted (Figure 1D and E). In addition, underlying liver cirrhosis with splenomegaly was observed. Percutaneous transhepatic gallbladder drainage (PTGBD) and cholangiography were performed. Cholecystography demonstrated contrast leakage from the gallbladder (Figure 1F).

He had a medical history of several years of alcoholic liver cirrhosis with mild esophageal varices and multiple gallbladder stones. Two months before the current attack, he underwent an abdominal CT scan to evaluate liver cirrhosis. At that time, CT revealed multiple stones in the gallbladder, without complications (Figure 1G).

DISCUSSION

Laparoscopic cholecystectomy has become a popular alternative to open surgery for the treatment of gallstones. With the increase in laparoscopic cholecystectomy, the incidence of gallstone spillage has increased, with an incidence ranging from 6% to 40%[1,2]. The complications of peritoneal spilled gallstones are abscess, fistula formation within various intraperitoneal organs, or sinus tract formation[3-7]. To the best of our knowledge, however, gallstone spillage caused by spontaneously perforated hemorrhagic cholecystitis in a patient who did not undergo cholecystectomy has not been reported.

The proposed mechanism of gallbladder perforation is stone impaction in the cystic duct, which leads to retention of secretion from mucus glands and distention, with progressive distention leading to vascular compromise, followed by necrosis and perforation[8]. During this process, bleeding can occur, which results in hemorrhagic chole-

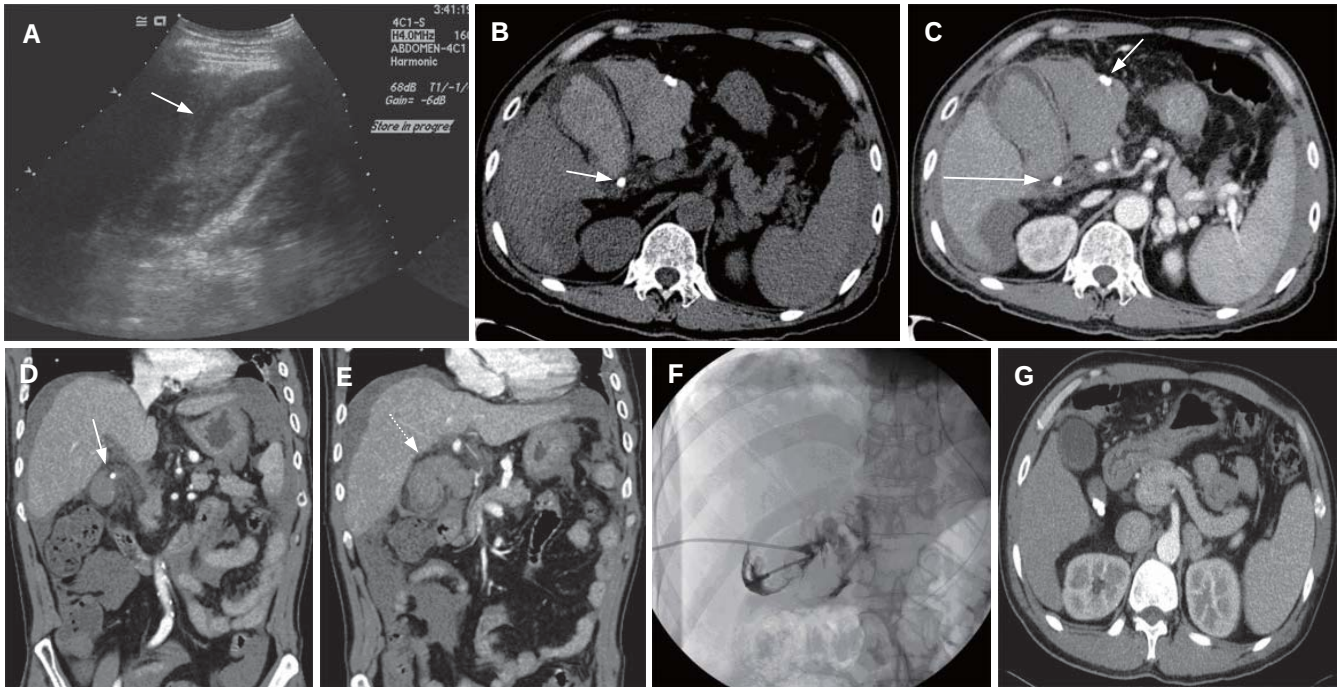

Figure 1 A 55-year-old man with right upper quadrant pain. US images (**A**) demonstrate heterogeneous, highly echogenic material, both within and outside the gallbladder lumen (arrows), with a positive sonographic Murphy's sign. Non-contrast (**B**) and contrast-enhanced (**C**) transverse CT images show high-attenuation (46-61HU) material, both in the gallbladder lumen and pericholecystic space. One stone is seen in the cystic duct (long arrow) and calcified material (with the same appearance as the cystic duct stone) is seen in the fluid collected (short arrow) around the gallbladder. Contrast-enhanced coronal CT images (**D**, **E**) show well the impacted cystic-duct stone (arrow), and the mucosal defect with continuation of hemorrhage (dotted arrow). PTGBD (**F**) with cholecystography demonstrates contrast leakage from the gallbladder. Contrast-enhanced transverse CT images (**G**) taken 2 mo before the current attack show multiple stones in the gallbladder neck without complications.

cystitis with hemoperitoneum. In our case, the patient had liver cirrhosis and therefore the risk of bleeding could have been increased.

In our study, US showed heterogeneous, highly echogenic material, both within and outside the gallbladder lumen, which may have been suggestive of gallbladder perforation with hemorrhage. However, we could not detect the exact perforation site nor spilled gallstones on US, and so we were not able to diagnose gallbladder perforation at that time. CT clearly demonstrated the perforation site at the gallbladder wall and spilled radiopaque stones that were missed on US. It has been reported that distended gallbladder, thickened and bulging gallbladder wall, pericholecystic fluid, cholelethiasis, and gallbladder wall defects are the US and CT findings of gallbladder perforation. The most specific of these findings is gallbladder wall defects, with a detection rate of 38.4% on US and 69.2% on CT[9]. The most common site of perforation is reported to be the fundus (70% of cases), due to poor vascular supply[10].

In conclusion, gallstone spillage due to spontaneous perforation is a very rare condition. However, US or CT visualization of mucosal disruption of the gallbladder wall, with gallstones within hemoperitoneum is suggestive of the condition.

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CASE REPORT

Mirizzi syndrome in an anomalous cystic duct: A case report

Cheol Woong Jung, Byung Wook Min, Tae Jin Song, Gil Soo Son, Hong Sik Lee, Seung Joo Kim, Jun Won Um

Cheol Woong Jung, Byung Wook Min, Tae Jin Song, Gil Soo Son, Seung Joo Kim, Jun Won Um, Department of Surgery, Korea University College of Medicine, 126-1, 5-Ga Anam-Dong, Sungbuk-Gu, Seoul 136-705, Korea
Hong Sik Lee, Department of Internal Medicine, Korea University College of Medicine, 126-1, 5-Ga Anam-Dong, Sungbuk-Gu, Seoul 136-705, Korea
Correspondence to: Jun Won Um, MD, Department of Surgery, Korea University Medical Center-Ansan Hospital, Korea University College of Medicine, 516 Gojan-Dong, Danwon-Gu, Ansan City, Kyungki-Do 425-707, Korea. junwonum@korea.ac.kr
Telephone: +82-31-4125952 Fax: +82-31-4134829
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Abstract

Mirizzi syndrome is a rare complication of gallstone disease, and results in partial obstruction of the common bile duct or a cholecystobiliary fistula. Moreover, congenital anatomical variants of the cystic duct are common, occurring in 18%-23% of cases, but Mirizzi syndrome underlying an anomalous cystic duct is an important clinical consideration. Here, we present an unusual case of type I Mirizzi syndrome with an uncommon anomalous cystic duct, namely, a low lateral insertion of the cystic duct with a common sheath of cystic duct and common bile duct.

Key words: Bile duct diseases and surgery; Cholelithiasis; Cholelithiasis and surgery; Cystic duct; Cholangiography

Jung CW, Min BW, Song TJ, Son GS, Lee HS, Kim SJ, Um JW. Mirizzi syndrome in an anomalous cystic duct: A case report. *World J Gastroenterol* 2007; 13(41): 5527-5529

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INTRODUCTION

Mirizzi described a functional hepatic syndrome in 1948, which consists of obstruction of the common hepatic duct secondary to compression by an impacted gallstone in the cystic duct or in the infundibula of the gallbladder (GB) associated with an inflammatory response involving the cystic duct and the common bile duct (CBD), surrounding inflammation, recurrent cholangitis, and spasm of the circular muscular sphincter in the hepatic duct[1]. Mirizzi syndrome (MS) indicates a narrowing of the common bile duct by a gallstone impacted in the cystic duct or a cholecystobiliary fistula. Many cases have so far been reported, and various operations have been suggested depending on the types of MS. Laparoscopic cholecystectomy has become the standard operation for gallstones, and many authors have adopted this operation for type I MS. However, high rates of conversion and bile duct injury indicate that this is not a safe treatment modality for MS, especially when combined with a cystic duct anomaly[2-5]. In this report, we present a case of type I Mirizzi syndrome, complicated by a rare anomalous cystic duct, which was operated with an open procedure.

CASE REPORT

A 34-year-old Korean female presented at our hospital complaining of abdominal pain of 2-d duration. The finding of her physical examination was not remarkable except for tenderness at the right upper abdominal quadrant with a positive Murphy sign. Furthermore, her laboratory findings were not remarkable except for an abnormal liver function: 2.1/1.6 mg/dL bilirubin (total/direct), 126 IU/L alkaline phosphatase, 310 IU/L aspartate aminotransferase, 625 IU/L alanine aminotransferase, and 114 IU/L gamma glutamyl transpeptidase. Ultrasonography (US) revealed multiple gallstones with GB wall thickening and a suspicious 1 cm-sized distal CBD stone. The same findings were also noted on endoscopic ultrasonography (EUS). Endoscopic retrograde cholangiopancreatography (ERCP) was conducted for a closer examination and removal of the CBD stone. However, ERCP showed that the 1 cm-sized gallstone was impacted in an anomalous cystic duct joined with the distal CBD. In addition, a gallstone compressed the CBD at the level of union between the pancreatic and biliary ducts (Figure 1). Mirizzi syndrome with low medial insertion of the cystic duct was preoperatively diagnosed, and endoscopic nasobiliary drainage (ENBD) tube was placed to decompress the biliary obstruction after endoscopic sphincterotomy.

During laparotomy via a right subcostal incision, the GB was found to be shrunken, thickened, and inflamed. The long and dilated cystic duct seen on ERCP was not identified in the operative field, and the GB was strongly attached to the CBD because of chronic inflammation and also possible anomalous union to the CBD (i.e., possibly due to a common sheath cystic duct and CBD). After subtotal cholecystectomy, the remnant of cystic duct was thickened and dilated to about 1.5 cm in diameter. Intraoperative choledochoscopy was performed *via* the

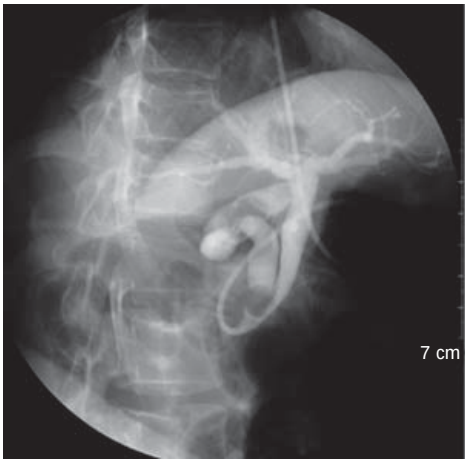

Figure 1 ENBD cholangiogam after endoscopic retrograde cholangiography showing a 1 cm-sized gall stone impacted in a cystic duct joining the extreme lower CBD portion and other stones in gall bladder. Mild CBD dilation was noted due to the presence of a cystic duct stone.

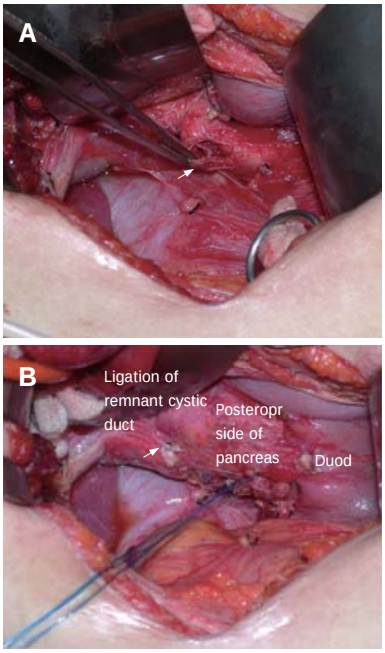

Figure 2 Torn pancreas and intrapancreatic portion of the cystic duct (**A**) and their primary repair (**B**) during lithotripsy of the impacted stone.

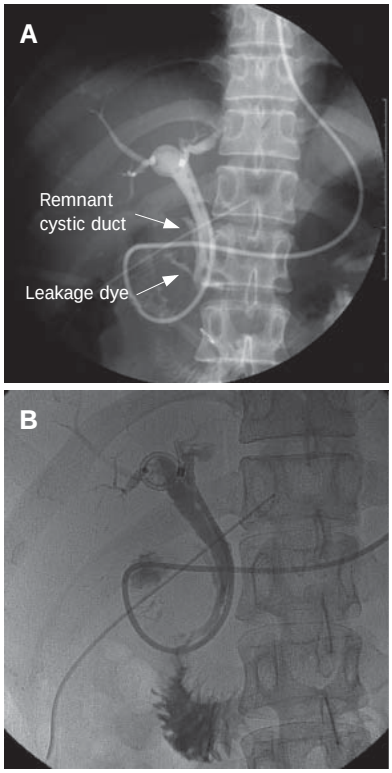

Figure 3 Follow-up ENBD cholangiogram showing minor leakage from the repaired cystic duct on the 7th postoperative day (**A**), but no visible leakage on the 21st postoperative day (**B**).

remnant of cystic duct and the impacted cystic duct stone was visualized. However, lithotripsy with choledochoscopy failed at this time. Another attempt was made to remove the cystic duct stone after duodenum mobilization through the remnant of cystic duct using a stone forceps made. Moreover, during this procedure, the pancreas and intrapancreatic portion of the cystic duct were torn, and the impacted cholesterol stone escaped retroperitoneally from the lacerated intrapancreatic cystic duct and pancreas (Figure 2A). After ligation of the stump of remaining cystic duct and primary repair of the torn cystic duct and pancreas (Figure 2B), intraoperative cholangiography *via* an ENBD tube showed neither leakage nor residual stone. A closed suction type drain was placed in the liver bed and retroduodenal space, and the abdominal wall was then closed. Because the ENBD catheter and endoscopic sphincterotomy were preoperatively performed, they could have played a role in biliary drainage in place of choledochotomy and T-tube insertion. The postoperative course was uneventful, except for minimal drainage from the closed suction drain and minor leakage from the repaired cystic duct, which was visible on the 7th postoperative day on ENBD cholangiography (Figure 3A). After conservative management, no visible leakage was observed from the repaired cystic duct on follow-up ENBD cholangiography on the 21st postoperative day (Figure 3B). After removal of the ENBD catheter, the patient was discharged on the postoperative 23rd d, and has now been doing well for over 3-years.

DISCUSSION

Congenital anatomical variants of the cystic duct are common, occurring in 18%-23% of cases. Among those cases, the cystic duct inserts into the middle third of the extrahepatic bile duct in 75% of cases and into the distal third in 10% of cases. Five types of cystic duct anomaly have been described: a long cystic duct with low fusion with the CHD, abnormally high fusion between a cystic duct and the CHD, accessory hepatic duct, cystic duct entering the right hepatic duct, and cholecystohepatic duct[6-8]. In particular, low medial insertion of the cystic duct deserves special attention, because this anatomical variant may lead to misdiagnosis by imaging studies, thus adversely affecting therapeutic intervention. In addition, anomalous cystic duct may also be a problem at cholecystectomy[9]. In the presently described case, ERCP demonstrated that the biliary obstruction was caused by a cystic duct gallstone in a low medially inserting cystic duct which joined the distal CBD near the ampulla of Vater, but not by a distal CBD stone as indicated by US and EUS.

Zhou[10] reported that 65 (5.9%) among 1100 cases had an abnormal cystic duct and common bile duct

confluence, as determined by ERCP, and he divided cystic duct anomalies into three types, and the very low-sited confluence was found in 9 (13.8%) cases among anomalous cystic ducts. Another study of 50 patients reported a single case (2%) of intrapancreatic confluence[11].

MS is a rare disease entity that accounts for about 0.1%-2.5% of all operations performed for gall bladder stones[12,13]. In 1982, McSherry *et al*[14] classified MS into two types, based on ERCP findings. Type I involves external compression of the common hepatic duct by a large stone impacted in the cystic duct, or the Hartmann's pouch, without any lesion in the gall bladder or common hepatic duct wall. In type Ⅱ, a cholecystocholedochal fistula is present, which is caused by a calculus that has eroded partly or completely into the common duct. In 1989, Csendes *et al*[12] classified MS into four types, and their classification categorized cholecystocholedochal fistula further depending on its extent of destruction. The McSherry classification or the Csendes classification has usually been used by clinicians, because these classifications more usefully guide surgical management.

MS has been highlighted because of its high incidence of iatrogenic biliary injuries and its demand for complex surgical procedures. Preoperative diagnosis of MS is difficult. However, it is important to prevent unexpected intraoperative morbidities, such as bile duct injury.

In the present case, a cystic duct stone was misdiagnosed as a CBD stone by US and EUS. However, in order to evaluate and remove the stone, ERCP was carried out and finally, a cystic duct stone with MS type I combined with a low lying anomalous cystic duct was diagnosed before surgery. Most MS cases have CBD obstruction symptoms such as jaundice (76.5%), which induce surgeons to attend to CBD problems[2]. However, cases not associated with CBD obstruction symptoms may be diagnosed as GB stone requiring only laparoscopic cholecystectomy. In a series reported by Tan *et al*[5], bile duct injuries were observed in 4 (16.7%) of 24 operatively treated patients, and all the 4 injuries occurred in patients without a preoperative diagnosis.

Surgical treatment of MS depends on its type. Although laparoscopic cholecystectomy has almost completely replaced open cholecystectomy for the treatment of symptomatic gallstone disease, laparoscopic cholecystectomy is relatively hazardous in patients with MS, because safe dissection of Calot's triangle is difficult due to severe local inflammations and adhesions[4]. Al-Akeely *et al*[2] reported that 2 of 6 type I MS patients successfully underwent laparoscopic partial cholecystectomy with an endo-GIA stapler. However, the procedure was converted to an open procedure in the remaining 4 patients. Schafer *et al*[4] reported that conversion to an open approach was needed in 24 of 34 patients (74%) with type I MS and in all 5 patients with type Ⅱ MS.

In the present case, open cholecystectomy was performed for MS combined with a cystic duct anomaly. However, bile duct injury occurred during removal of the impacted cystic duct stone. Thus, we would like to advise that, if MS combined with a cystic duct anomaly is diagnosed before surgery, the operator should not hesitate to perform open surgery and dissect carefully, while considering anatomical deformities associated with chronic inflammation. Moreover, intraoperative cholangiography should be performed to minimize the risk of biliary injury.

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CASE REPORT

Hepatic abscess secondary to a rosemary twig migrating from the stomach into the liver

Aleksandar R Karamarkovic, Srdjan P Djuranovic, Nada P Popovic, Vesna D Bumbasirevic, Ana D Sijacki, Ivan V Blazic

Aleksandar R Karamarkovic, Ana D Sijacki, Ivan V Blazic, Center for Emergency Surgery, Clinical Center of Serbia, School of Medicine, University of Belgrade, Belgrade, Serbia
Srdjan P Djuranovic, Clinic for Gastroenterology and Hepatology, Clinical Center of Serbia, School of Medicine, University of Belgrade, Belgrade, Serbia
Nada N Popovic, Vesna M Bumbasirevic, Institute of Anaesthesiology, Clinical Center of Serbia, School of Medicine, University of Belgrade, Belgrade, Serbia
Correspondence to: Aleksandar R Karamarkovic, MD, PhD, Associate Professor, Head of Department of Surgery Ⅲ, University Center for Emergency Surgery, Clinical Center of Serbia, School of Medicine, University of Belgrade, Pasteur Str.2, Belgrade 11000, Serbia. smnm@Eunet.yu
Telephone: +381-64-1279250 Fax: +381-11-3065604
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Abstract

The ingestion of a foreign body that penetrates the gastric wall and migrates to the liver, where it causes an abscess is uncommon. A case of an ingested rosemary twig perforating the gastric antrum, then migrating to the liver, complicated by hepatic abscess and Staphylococcus aureus sepsis is reported. A 59-year-old man without a history of foreign body ingestion was admitted to our hospital because of sepsis and epigastralgia, which had progressively worsened. No foreign body was identified at preoperative imaging, but a rosemary twig was discovered during laparotomy. The liver abscess and sepsis were controlled successfully with surgery and antibiotics. This unusual condition should be kept in mind when dealing with cases of hepatic abscess, or even sepsis of unknown origin. Despite the improvement of non-surgical techniques such as percutaneous drainage and interventional endoscopy, surgery still remains important in the treatment of hepatic abscess caused by an ingested foreign body.

Key words: Foreign Body; Gastrointestinal perforation; Hepatic Abscess; Ingestion; Migration

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INTRODUCTION

The first case of hepatic abscess as a result of gastrointestinal tract (GIT) perforation caused by a foreign body was published by Lambert in 1898[1]. Most foreign bodies pass through the GIT without causing any damage once they pass the lower esophageal sphincter[2]. It is not unusual to come across patients in clinical practice with GIT perforation due to ingested foreign bodies, but the development of a secondary hepatic abscess due to foreign body perforation of the gastric wall is a rare condition[1-4]. In the majority of cases, an early diagnosis is difficult to make because of the non-specific clinical presentation[3].

CASE REPORT

A 59-year-old man presented with epigastric, right upper abdominal pain and intermittent high-grade fever, with chills and rigors for the past 2 mo. There was no history of foreign body ingestion. He had received treatment for his fever of unknown origin at a district hospital in the form of antibiotics (ceftriaxone, 2.0 g daily) and antipyretics. On admission, examination revealed a septic, high-grade febrile patient (38.9℃) with tachycardia (pulse 128 bpm) and tachypnoea (21 breaths/min). The white blood cell count was 28.8 × 10^9/L, alkaline phosphatase was 180 U/L, bilirubin was normal, and C-reactive protein (CRP) was 320 mg/L. The abdomen was tense with tenderness in the epigastrium and right hypochondrium, without any signs of peritoneal reaction. Chest X-ray revealed a right-sided pleural effusion, but X-ray of the abdomen was normal. Ultrasound (US) examination, contrast-enhanced computerized tomography (CT) (Figure 1) and magnetic resonance imaging (MRI) of the abdomen (Figure 2) revealed a hepatic abscess of 11.7 cm × 10.3 cm × 8.8 cm in the segments S4b-S5. The patient was started on meropenem and subjected to exploratory laparotomy, which revealed a huge abscess occupying the central segments of the liver, and concomitant acute calculous cholecystitis. There was no association between the inflammatory process in the gallbladder and abscess formation. After cholecystectomy, hepatotomy along the gallbladder bed was performed, and about 500 mL of pus

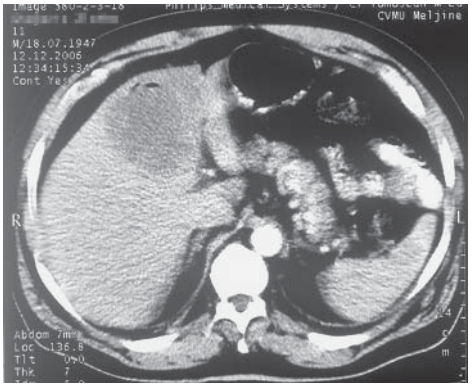

Figure 1 CT scan of the abdomen demonstrating a liver abscess containing a small amount of gas.

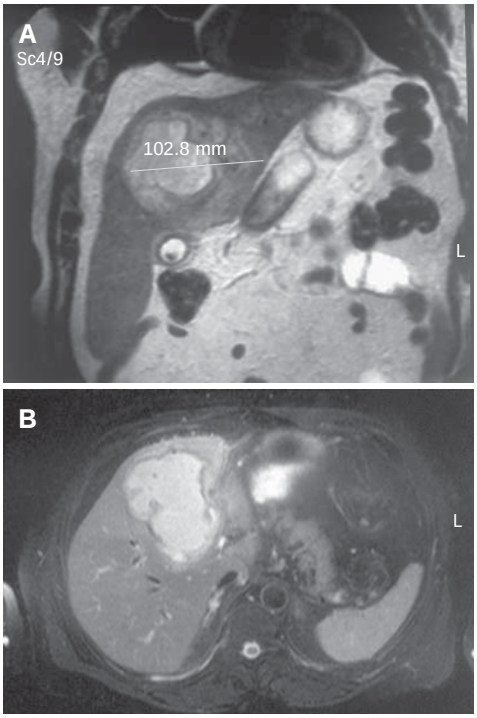

Figure 2 MRI of the abdomen showing the abscess in liver segments S4b-S5. (**A**) T2W coronal view; (**B**) T2W axial view.

was drained and a rosemary twig of 4.5 cm was retrieved from the abscess cavity (Figure 3). Since there was no obvious fistulous communication between the liver and stomach or duodenum, a careful examination of the upper GIT revealed a small covered perforation of the gastric antrum wall. The perforation was repaired by using single-layer interrupted sutures (Figure 4). The abscess was also drained with a triple-tube lavage system. Vancomycin was added postoperatively due to subsequent blood culture that showed Staphylococcus aureus. The patient recovered uneventfully and was discharged on postoperative d 10.

DISCUSSION

The reported incidence of foreign bodies penetrating the GIT is < 1%[1,2,5], with the objects being pointed or sharp in most cases, such as sewing needles, tooth picks, and

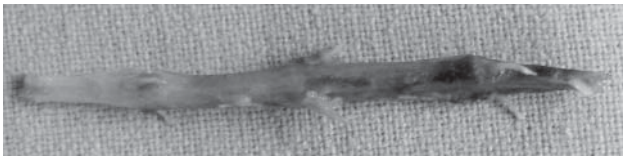

Figure 3 Rosemary twig after retrieval from the hepatic abscess.

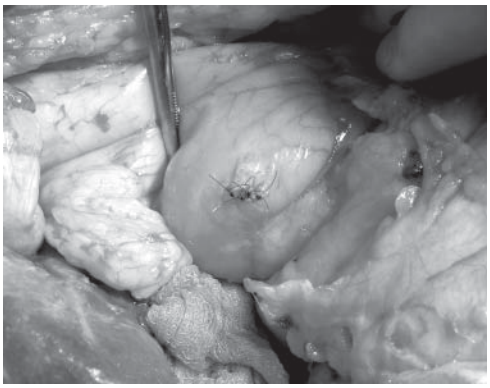

Figure 4 Repair of the perforation site in the gastric antrum.

chicken and fish bones[5-8], pens, toothbrushes and dental plates[9-11].

The most common sites of perforation of the GIT are the stomach and duodenum[1-3]. Abscess formation occurs more often on the left hemiliver[2,3]. The ingestion of a foreign body that penetrates the GIT wall and migrates to the liver, where it causes an abscess is indeed rare, with 46 cases being reported in the recent literature[3]. No report of hepatic abscess caused by ingestion of a rosemary twig (used for food flavoring), has been found so far in the medical literature.

The classical presenting features of hepatic abscess, such as fever with chills, abdominal pain and discomfort, and jaundice are present in only a small number of patients[1-4]. Most patients have non-specific symptoms such as anorexia, vomiting or weight loss with leucocytosis[6-9], or increased transaminases, bilirubin or alkaline phosphatase[10,11]. The migrating foreign body may remain silent for a long time and may only be discovered if there are features of infection or abscess formation[1]. The presentation of a penetrating foreign body (tooth pick) as a granulomatous liver abscess has been reported 1 mo after ingestion, requiring partial lateral resection of the liver[8]. The prolonged time course of the illness, the lack of history of foreign body ingestion, the relatively non-specific symptoms and signs, and the non-specific results obtained by using conventional radiography have resulted in delayed recognition of this possibly fatal diseas [4]. Deaths caused by missed or delayed diagnosis have been reported, one of which was discovered on autopsy[12-14]. Thus, both a high clinical suspicion index and prompt treatment are necessary for this rare condition[1-3,11-15]. An ingested foreign body may be identified with plain X-rays of the abdomen, if the body is radio-opaque. Other methods of foreign body identification include US, CT, MRI, upper GIT endoscopy, colonoscopy,

and laparotomy[8,15-16]. Endoscopy may be helpful when performed early, before the foreign body migration and mucosal healing[3,7].

The recommended treatment is exploratory laparotomy to evacuate the hepatic abscess, remove the foreign body, and repair the perforation site in the GIT[1-3,16]. Since the gastric perforation is small and is probably covered by the omentum or hepatic lobe, minimally invasive treatment such as percutaneous drainage of the pus collection, combined with endoscopic removal of the foreign body, can be employed to reduce open surgery[2,9]. Also, the successful treatment of hepatic abscess and foreign body removal by the percutaneous transhepatic approach has been reported[10].

In conclusion, we report a very rare case of the migration of an ingested rosemary twig into the liver through the stomach, which resulted in hepatic abscess and sepsis. Due to the lack of obvious fistulous communication between the liver and upper GIT, careful exploration of the abscess cavity is of great importance. This condition should be kept in mind when dealing with cases of hepatic abscess, or even sepsis of unknown origin. Therefore, an early diagnosis and prompt intervention are optimal for treatment and necessary to avoid death. Despite the improvement of non-surgical techniques such as percutaneous drainage and interventional endoscopy, surgery still remains important in the treatment of hepatic abscess caused by an ingested foreign body.

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CASE REPORT

Carcinosarcoma of the stomach: A case report and review of the literature

Tomislav Randjelovic, Branka Filipovic, Darko Babic, Vesna Cemerikic, Branislav Filipovic

Tomislav Randjelovic, Department of Gastrointestinal Surgery, Clinical and Hospital Center "Bezanijska Kosa," Belgrade, Serbia
Branka Filipovic, Department of Gastroenterohepatology Clinical and Hospital Center "Bezanijska Kosa," Belgrade 11080, Serbia
Darko Babic, Department of Pathology, Clinical and Hospital Center "Bezanijska Kosa," Belgrade 11080, Serbia
Vesna Cemerikic, Laboratory for Histopathological and Cytological Diagnostics "Histolab," Belgrade 11080, Serbia
Branislav Filipovic, Institute of Anatomy, School of Medicine, University of Belgrade, Belgrade 11080, Serbia
Correspondence to: Branka Filipovic, MD, MSc, Department of Gastroenterohepatology, Clinical and Hospital Center "Bezanijska Kosa," Autoput bb, Belgrade 11080, Serbia. branaf@bitsyu.net
Telephone: +381-11-3010700 Fax: +381-11-3010751
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Abstract

Carcinosarcomas are rare, malignant, biphasic tumors. We report the case of a 62-year-old man with gastric carcinosarcoma, along with its clinical, macroscopic and histopathological features. Macroscopically, a specimen of deformed stomach was obtained that measured 200 mm × 150 mm × 100 mm. A 150 mm × 100 mm × 50 mm exophytic tumoral mass (Borrmann type Ⅰ) was found, which involved the posterior wall from the cardia to the antrum. Histopathologically, a mixed type of malignancy was revealed: an adenocarcinoma with intestinal metaplasia, with interposed fascicles of fusiform atypical cells and numerous large, rounded and oval cells. The tumor showed positive histochemistry for cytokeratin 18, epithelial membrane antigen, carcinoembryonic antigen, chromogranin A and vimentin. Liver metastases were diagnosed 8 mo postoperatively, and the patient died 4 mo later. A review of the available literature is also presented.

Key words: Carcinosarcoma; Histochemistry; Pathology; Stomach

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INTRODUCTION

Carcinosarcomas are rare, malignant, biphasic tumors. In the upper gastrointestinal tract, they are most frequently observed in the esophagus, while localization in the stomach has been less frequently reported[1-3]. We present the case of a 62-year-old man with gastric carcinosarcoma, along with its clinical, macroscopic and histopathological features.

CASE REPORT

The patient was a 62-year-old man admitted for surgery with a 2-mo history of blunt epigastric pain, nausea, loss of body weight and intermittent bleeding from the upper gastrointestinal tract. His and his family medical history was unremarkable.

Upon admission, the patient was in a forced position, bent anteriorly with a facial expression of pain. General examination revealed marked pallor, and tenderness in the epigastric region, radiating to the right side of the anterior abdominal wall. In the space of Labbé, an elastic, resistant, fixed mass was palpated. Routine laboratory parameters were found to be normal, except for markers of hypochromic anemia and inflammation: hemoglobin 100 g/L, hematocrit 26%, mean corpuscular volume (MCV) 78 fL, iron blood level 6.8 μmol/L, iron-binding capacity 84 μmol/L, saturation 8%, plasma fibrinogen 6.9 g/L, and erythrocyte sedimentation rate 85 mm at the end of the first hour (Wintrobe). The concentration of CA 72.4 was 110 U/mL.

Endoscopic examination revealed an exophytic, lobulated mass that infiltrated the entire posterior wall of the stomach, from the cardia to the antrum, obturating the gastric lumen throughout. An endoscopically taken biopsy revealed signs of carcinosarcoma, with strongly expressed adenomatous and fibromatous components. Barium-based contrast radiography revealed a satisfactorily passable pyloric canal, despite the initial antral obturation. Computerized abdominal tomography (Figure 1) detected an irregular inhomogeneous, prominent tumorous formation (120 mm × 80 mm × 50 mm) in the stomach, with enlarged solitary lymph nodes of up to 2 cm disseminated along the minor and major gastric curvatures. The patient subsequently underwent total gastrectomy with Roux-en-Y esophagojejunostomy and extirpation of the affected lymph nodes. Macroscopically, a specimen

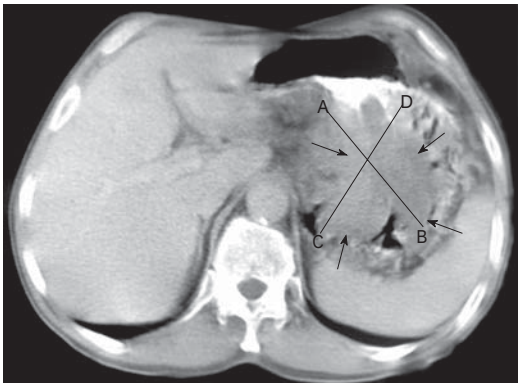

Figure 1 CT scan of the abdomen showing tumor (arrows). AB, CD: tumor diameters.

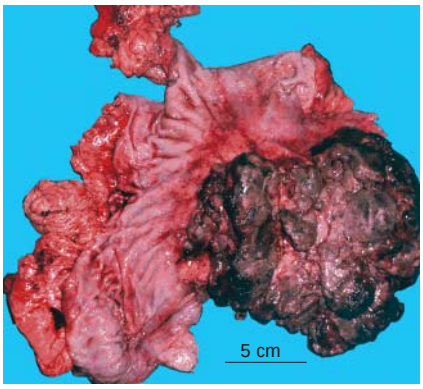

Figure 2 Macroscopic specimen obtained during surgery. Tumor underwent central necrosis, and a hemorrhagic zone is visible on the periphery.

of deformed stomach that measured 200 mm × 150 mm × 100 mm was obtained. A 150 mm × 100 mm × 50 mm exophytic tumoral mass (Borrmann type Ⅰ) was found, which involved the posterior wall from the cardia to the antrum. Areas of necrosis and haemorrhagia were observed in the tumor (Figure 2). The tumor did not invade the esophagus or duodenum, and metastases to other organs were not observed (TNM stage ⅢA).

Histopathologically, the tumor involved all the layers of the gastric wall. The malignancy had two components, epithelial and mesenchymal. The epithelial component consisted of irregular, dilated adenomatous structures, along with a low cylindrical epithelium, with atypical, pleomorphic hyperchromatotic or vesiculous nuclei and detectable nucleoli. Between the glandular formations spread the mesenchymal component, consisting of fascicles of fusiform atypical cells and numerous large, rounded and oval cells, with extremely pleomorphic, hyperchromatic nuclei. Among the aforementioned, multiple atypical, multinuclear giant cells with bizarre hyperchromatotic nuclei and spotted vacuolated cytoplasm were scattered (Figure 3A and B). The epithelial component showed positive histochemistry for cytokeratin 18 (Figure 4), epithelial membrane antigen (EMA) and carcinoembryonic antigen (CEA). Chromogranin-A-positive epithelial cells were also observed. The mesenchymal component showed intensive staining for vimentin (Figure 5), although neither muscular nor neural differentiation was found. No *H pylori* was seen.

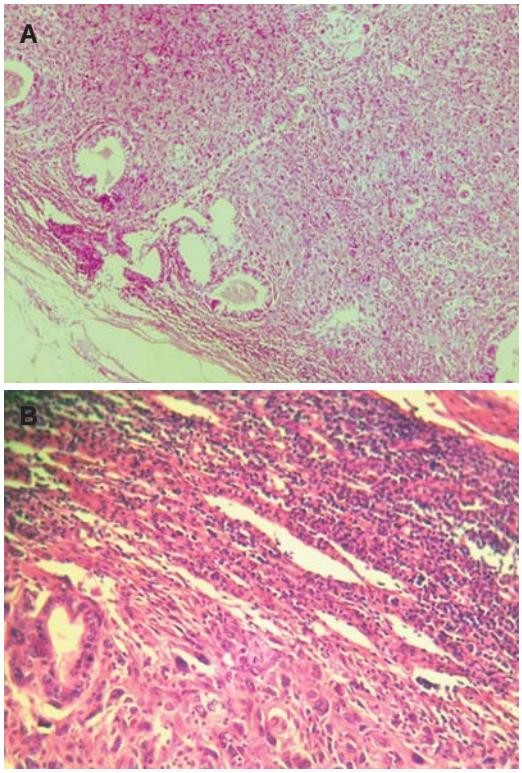

Figure 3 Massive lymph node infiltration with tumor cells. Only a few tubules can be seen on the lymph-node periphery. In between and all around, large polygonal cells are haphazardly arranged (sarcomatous component). This appearance resembles that of the main tumor mass. **A**: hematoxylin-eosin (HE), × 50; **B**: HE, × 100.

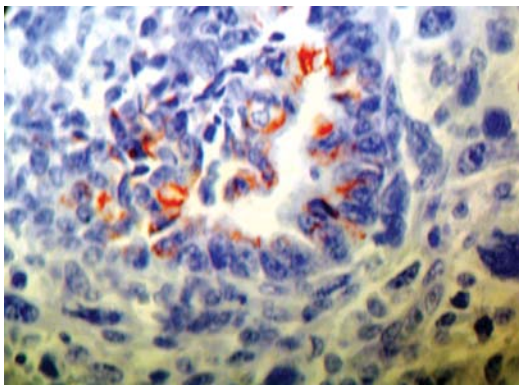

Figure 4 Cytokeratin-18-positive epithelial cells arranged in tubules (× 400).

The patient was discharged on the fifteenth postoperative day in a very well condition. Eight months after operation, liver metastases were observed on CT scanning, but his Karnofsky performance status (50) and Eastern Cooperative Oncology Group performance status (3) did not allow the administration of chemotherapy, and therefore he only received symptomatic medications. He died about 4 mo later.

DISCUSSION

In this paper, we presented the case of a patient with stomach carcinosarcoma, with simultaneous occurrence of moderately to well-differentiated adenocarcinoma

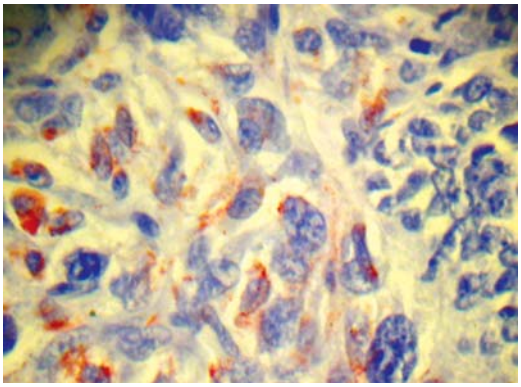

Figure 5 Vimentin-positive polygonal tumor cells (× 400).

and traces of neuroendocrinous, chromogranin-A-positive elements, combined with a vimentin-positive mesenchymal component. To the best of our knowledge, this is the first case of gastric carcinosarcoma seen in this part of the world. According to the available sources, about 50 cases of gastric carcinosarcoma have been reported so far, mostly in Japan and predominantly in the male population, mostly over the age of 60 years[4-6]. An Italian group has experienced five cases of synchronous occurrence of adenocarcinoma and stromal tumor during a 10-year period[7]. Carcinosarcomas in the stomach may be polypoid, exophytic or endophytic, with generally ulcerated surfaces, and they frequently infiltrate the gastric wall in the antral or pyloric region, and form large tumor masses[2,4,7]. Intestinal adenocarcinoma is predominant, but carcinoid and endocrinous or neuroendocrinous elements have been observed during the synchronous appearance of carcinoma and sarcoma in the stomach, while the sarcomatous component can vary between myoblastic, rhabdomyoblastic, chondroblastic and osteoblastic differ entiation[2,5,6,8-11]. Metastasis of carcinosarcoma, however, may be entirely carcinomatous, sarcomatous or biphasic in appearance[4].

Immunocytochemistry seems to be the gold standard for diagnosis of carcinosarcoma, because contrast-based radiography, computerized tomography (CT) and even endoscopy appear to be less efficient, and occasionally, even standard light microscopy is not adequate. Therefore, CEA, EMA, pancreatin, chromogranin A, CD56 and synaptophysin staining are highly specific markers for the carcinomatous components, while desmin, vimentin and α-smooth muscle/sarcomeric actin show affinity for the sarcomatous elements[5,12].

Therapy of carcinosarcoma is always radical and comprises partial or total gastrectomy with Roux-en-Y deviation of one of the jejunal loops, although some complications might appear in the postoperative period[13,14]. Some experimental studies reported possible tumor reduction following treatment with methionine/valine-depleted enteral nutrition, although its efficacy in humans is ambiguous and remains to be established[15].

Prognosis of carcinosarcoma in the stomach is poor[6], and patients with gastric endocrine cell carcinoma have a poorer prognosis than those with other types of gastric carcinoma. The mean survival period is estimated to be 10-15 mo, and overall tumor recurrence in the first postoperative year is greater than 50%[5,9,12].

With respect to the histogenesis of carcinosarcoma, two hypotheses have been proposed. Some authors have suggested that carcinosarcoma is derived from a single totipotential stem cell that has the ability to pursue both epithelial and mesenchymal differentiation[14]. There is no strong evidence that *H pylori* infection influences the appearance of carcinosarcoma[7,14].

In conclusion, carcinosarcoma of the stomach is a rare tumor with high malignant potential, often of unclear etiology. At present, no clinical tests are available for early diagnosis (MRI, barium-based gastrography). The gold standard for definitive diagnosis is immunohistochemical staining of endoscopic biopsy. The possibilities for therapy are confined to radical Roux-en-Y esophagojejunostomy, and recurrence of the tumor can be expected within the first postoperative year.

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CASE REPORT

Perivascular epithelioid cell tumor of the liver: A report of two cases and review of the literature

Song-Hua Fang, Li-Na Zhou, Mei Jin, Ji-Bo Hu

Song-Hua Fang, Li-Na Zhou, Mei Jin, Ji-Bo Hu, Department of Radiology, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou 310016, Zhejiang Province, China
Correspondence to: Ji-Bo Hu, Department of Radiology, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou 310016, Zhejiang Province , China. fangsonghua@163.com
Telephone: +86-571-86006765 Fax: +86-571-86044817
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Abstract

Perivascular epithelioid cell tumor (PEComa) is a rare tumor which arises from mesenchymal tissues. It is predominant in the uterus, but very rare in the liver. To the best of our knowledge, less than 5 cases of PEComa of the liver have been reported. Herein we present two pathologically proven cases of PEComa of the liver, retrospectively analyze their clinical and imaging features, and review the literature.

Key words: Liver; Neoplasm; Tomography, X-ray computed; Magnetic resonance imaging

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INTRODUCTION

Perivascular epithelioid cell tumor (PEComa) is a rare tumor which arises from mesenchymal tissues. The uterus is the predominant site, but it is very rare in the liver[1-3]. To the best of our knowledge, less than 5 cases of PEComa of the liver have been reported[2,3]. Herein we present two pathologically proven cases of PEComa of the liver, retrospectively analyze their clinical and imaging features, and review the literature.

CASE REPORT

Case 1

A 56-year-old woman complained of abdominal distention for a week. The laboratory examinations were normal. Ultrasonography found a mass in the superior segment of left lateral lobe of the liver. Non-enhancement CT showed a round isodense mass in the Ⅳ segment of the left liver with ill-demarcated margin. There was no evidence of fatty density, calcification and necrosis in the mass. On contrast enhancement CT, a well demarcated mass, sized 5.1 cm × 4.2 cm, was found with significant and heterogeneous enhancement (Figure 1A). It was more strikingly enhanced on portal venous phase than on arterial phase. Focal nodular hyperplasia (FNH) or adenoma of the left liver was considered before operation. After operation, pathological diagnosis was established as PEComa of the left liver (Figure 1B). Neither primary recurrence nor metastasis was found during the 2-year follow-up.

Case 2

A 63-year-old woman was found to have a mass of liver incidentally in physical examination. Blood, stool and urine routines were normal. Pre-contrast CT scan revealed a lower density mass in the lobus caudatus with a well demarcated margin and homogeneous density. Contrast-enhanced CT showed significantly and heterogeneously patchy enhancement of the lesion on arterial phase (Figure 2A), being slightly hypodense on delayed CT scan. On T1-weighted images, a round homogeneous hypointense mass in the lobus caudatus was found. On T2-weighted images (Figure 2B), the mass had mildly heterogeneous high-signal intensity with a well-demarcated margin. After contrast enhancement, the mass had striking and homogenous enhancement on arterial phase and venous phase. The diagnosis was FNH or HCC of the liver before operation. The gross appearance of the tumor was a smooth, grey and brown lesion with a capsule. The tumor cells were polygonal with eosinophilic cytoplasm. The final diagnosis was PEComa of the liver. Neither primary recurrence nor metastasis could be found during the 1-year follow-up.

DISCUSSION

The term "PEComa" was introduced by Zamboni *et al* in 1996[4]. In 2002 and 2003, two monographs published under the auspices of the World Health Organization (WHO) recognized a family of neoplasm with perivascular epithelioid cell differentiation and accepted the designation "PEComa"[5]. In the WHO soft tissue volume, PEComas are defined as "mesenchymal tumors composed of histologically and immunohistochemically distinctive

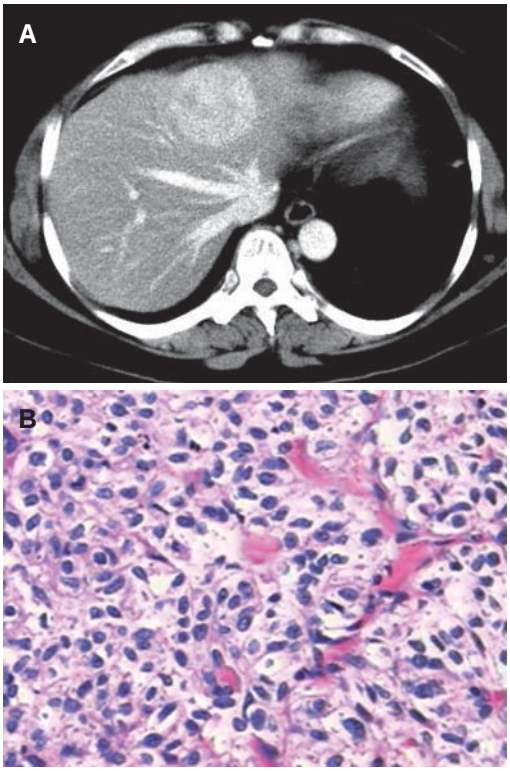

Figure 1 The PEComa of the liver in a 56-year-old woman. **A**: Contrast-enhanced CT scan shows significant and heterogeneous enhancement of the lesion; **B**: Photomicrograph shows polygonal or short spindle cells with oval nuclei and clear abundant cytoplasm (HE, × 200).

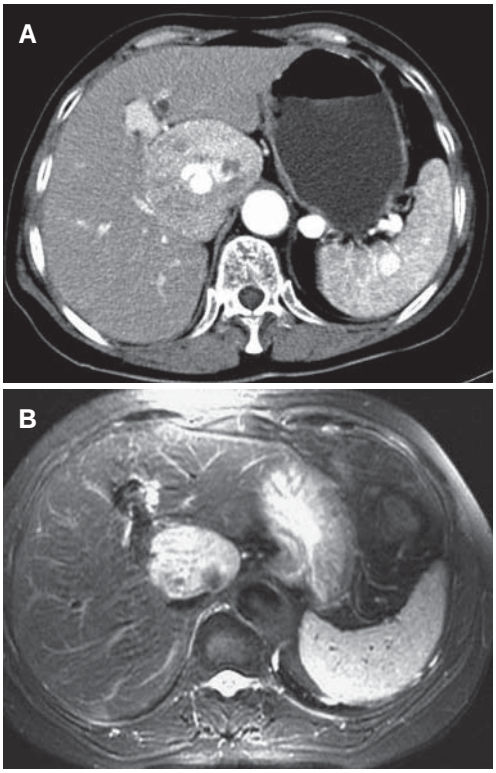

Figure 2 The PEComa in the lobus caudatus of the liver in a 63-year-old woman. **A**: Contrast-enhanced CT scan demonstrates a tumor in the lobus caudatus; **B**: Axial T2-weighted (7058/84) MR image shows a hyperintense mass with well-demarcated tumor margins.

perivascular epithelioid cells (PECs)"[5].

PECs are characterized by perivascular location, often with a radial arrangement of cells around the vascular lumen. Typically, PECs in an immediate perivascular location are most epithelioid and spindle cells resembling smooth muscle are seen away from vessels[5]. The PEC is characterized by positivity with melanocytic markers, such as HMB-45, Melan-A and microphthalmia transcription factor. Desmin is less often positive and cytokeratin and S100 protein are usually absent[5].

PEComas have been reported in the uterus, falciform ligament, gastrointestinal tract, kidney, pancreas, pelvic sidewall, skull, vulva, prostate, thigh, common bile duct and heart. It now appears that PEComas may potentially arise from any anatomic location, but the uterus is the predominant site. Of the 51 cases of PEComa that have been documented in the literature, 46% (21/51) were described in the uterine corpus and 90% developed in females[5]. The PEComas of the liver are extremely rare, and only 5 cases have been reported to date[6].

Clinical presentation of hepatic PEComa has no specificity. It is often found accidentally in physical examinations. But in other organs, the clinical presentation of PEComa might be significant, for example, uterine PEComa may induce uterine bleeding strikingly. Sometimes, a mass can be touched in the PEComa of the lower digestive system and soft tissues.

It is very difficult to make correct diagnosis of PEComa of the liver preoperatively. It is often misdiagnosed as hepatocellular carcinoma, hemangioma, FNH, adenoma and angiomyolipoma. Imaging modalities may be useful because they can help to differentiate PEComa from hepatocellular carcinoma. But the final diagnosis can only be made by pathology.

Clear criteria for malignancy in PEComas have not been elaborated, due to their rarity[5], but there have been a few reports about the tumor metastases[6,7]. On the basis of prior reports, it appears that PEComas displaying any combination of infiltrative growth, marked hypercellularity, nuclear enlargement and hyperchromasia, high mitotic activity, atypical mitotic figures, and coagulative necrosis should be regarded as malignant[5,8]. Malignant PEComas are aggressive sarcomas that frequently result in the death of affected patients, therefore, a close and long-term clinical follow-up is suggested.

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LETTERS TO THE EDITOR

Unusual colonoscopy finding: *Taenia saginata* proglottid

Nayan M Patel, Eric L Tatar

Nayan M Patel, Eric L Tatar, University of Medicine and Dentistry of NJ, Robert Wood Johnson Medical School, Department of Internal Medicine, New Brunswick, NJ 08901, United States
Correspondence to: Nayan M Patel, University of Medicine and Dentistry of NJ, Robert Wood Johnson Medical School, Department of Internal Medicine, New Brunswick, NJ 08901, United States. nayan814@hotmail.com
Telephone: +1-732-9913606
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Abstract

Infection with tapeworms is a major problem in many parts of the world. Patients may be asymptomatic or have a significant morbidity depending on the species. Infection with *Taenia* species is sometimes found by expulsion of eggs or proglottids in stool. Species specific diagnosis of *Taenia* is difficult, but possible. We present a case of *Taenia saginata* incidentally discovered, and risk factors for transmission, diagnosis, symptoms, and treatment.

Key words: *Taenia saginata*; colonoscopy; Tapeworm

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TO THE EDITOR

A 63-year-old Lebanese male presented for routine surveillance colonoscopy of polyps. He denied abdominal pain, hematochezia, weight loss, or change in bowel habits. Physical examination and laboratory studies including a complete blood count were normal. While withdrawing the scope, the following item was seen (Figure 1).

The linear white object represented a parasite. On endoscopic examination, it was found to move within the colon. When retrieved and further analyzed, it was found to be a proglottid of *Taenia saginata*, the beef tapeworm. The scolex and majority of the worm are present in the small bowel, with the head usually residing in the jejunum or ileum. Each segment, known as a proglottid, has a complete set of reproductive organs. The adult worm may have hundreds to thousands of proglottids. The more distal the proglottids are, the more mature they are, containing an increasing number of eggs. *Taenia* species bud off distal segments from the rest of the body that are passed through the feces. The mature *T. saginata* tapeworm can reach 4-6 meters or more in length, and has 1000-2000 proglottids. The scolex has 4 suckers, but no hooks. In contrast, the mature T. solium, or pork tapeworm can reach 2-4 meters or more in length, and has 800-1000 proglottids. The scolex has 4 suckers and a small rostellum with a double crown of 25-30 small hooks[1].

Finding eggs or proglottids in the stool makes the diagnosis of *Taenia* infection. The eggs of *T. saginata* are indistinguishable from *T. solium*, and a species specific diagnosis requires examination of a proglottid segment. The microscopic differentiation of gravid *T. saginata* proglottids (usually more than 15 lateral uterine branches, vaginal sphincter muscle, and two ovarian lobes) and *T. solium* proglottids (usually 5-10 uterine branches on each side, vaginal sphincter muscle absent, one ovarian lobe) is possible. This is the only practical method that can be used in a basic laboratory if only gravid proglottids passed out in stool are present for diagnosis. The presence of a vaginal sphincter muscle in the proglottid can identify the organism as *T. saginata*. The presence of 2 ovarian lobes is also a specific trait of *T. saginata*. Following antihelminth therapy, the scolex is shed and in some cases may be retrieved. The absence of hooks on the scolex is a characteristic of *T. saginata*[2]. If findings are doubtful, the differentiation should be done in a specialized helminthological laboratory by enzyme electrophoresis, polymerase chain reaction (PCR), or various immunological assays[3].

The beef tapeworm is a common infection of both humans and cattle throughout the world, particularly in areas wherever beef is eaten. Areas of high prevalence are sub-Saharan African, southeast Asia, and the Middle East. Infection is associated with eating raw beef, poor sanitation, and allowing cattle on pastures fertilized by sewage sludge contaminated with human feces[4]. Lifecycles for *Taenia* involve two mammalian hosts, a carnivorous or omnivorous host, and a herbivorous intermediate host. In the tapeworm life cycle, humans are the final host and infections are acquired by ingesting raw or undercooked meat containing the cysticercus stage in a host capsule. When the cysticercus stage reaches the stomach, proteolytic enzymes start dissolving the capsule. In the small intestine, the cysticercus is stimulated to evaginate. The scolex attaches to the intestinal mucosa by means of 4 suckers and starts growing into a mature tapeworm.

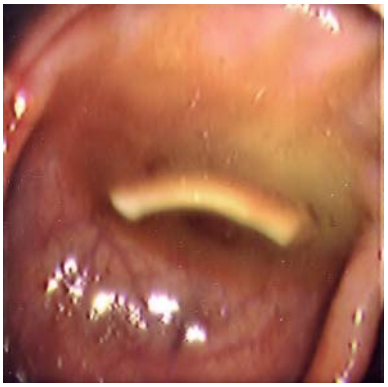

Figure 1 Endoscopic examination showing a parasite (a proglottid of *Taenia saginata*) moving in the colon.

Mature tapeworms have been known to live in the human gastrointestinal tract for up to 25 years[1]. Upon further questioning, our patient noted that he frequently ate raw beef in his native country.

Most patients who carry an adult *T. saginata* tapeworm are asymptomatic. The only symptom found in such a patient may be the spontaneous passage of proglottids. Nonspecific symptoms such as abdominal discomfort, epigastric pain, nausea, vomiting, diarrhea, and weight loss are known to occur[1], but these symptoms are rare. Obstruction of the appendix, pancreatic duct, or common bile duct by proglottid segments has been reported[1]. The occurrence of weight loss and malnutrition is extremely rare. B12 deficiency and megaloblastic anemia are not seen in *Taeniasis*, and associated with *Diphyllobothrium* species found in fish species. B12 deficiency is thought to occur as a result of the ability to compete with the human host for vitamin B12.

While the adult form of *T. saginata* generally does not lead to serious complications, juvenile forms of other tapeworm species may lead to such complications. Ingested ova from *T. solium*, the pork tapeworm, can form cysticercosi in the brain, subcutaneous tissue, skeletal muscle, eye, or other organs causing a significant morbidity. The life cycle of *T. solium* includes pigs that are the intermediate host because they develop the larval stage and transmit the parasite when human beings ingest insufficiently cooked pork[5]. It is endemic in Mexico, Latin America, tropical Africa, southeast Asia, the Philippines, and the Indian subcontinent[2]. Ova from *Echinococcus*, a tapeworm associated with canines, can also cause cysticercosi with a predominance for occurrence in the liver. There is no evidence that cysticerci can develop in humans as a result of T. saginata infection[6].

The treatment of choice in intestinal *Taeniasis* (*T. saginata* and *T. solium*) is praziquantel, a synthetic heterocyclic isoquinolone-pyrazine derivative. A single dose of 5 to 10 mg/kg has an efficacy of greater than 95%. Praziquantel induces ultrastructural changes in the teguments of parasites, resulting in increased permeability to calcium ions[7]. Calcium ions accumulate in parasite cytosol causing muscular contractions and ultimate paralysis of the worm. Additionally, this exposes parasite antigens to the host immune response. The ultimate response is dislodgement of worms from their intestinal sites and subsequent expulsion by peristalsis. For successful treatment, the scolex must be destroyed, and eliminated because residual scolex can result in regrowth. Albendazole or praziquantel can be used in the treatment of cysticercosis.

For people in high risk communities, primary prevention of *Taeniasis* is the removal of intermediate hosts such as cattle and pigs from the parasite's life cycle by eliminating exposure to raw sewage[1] and adequately cooking meats before consumption.

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Division of Gastroenterology and Hepatology, Graduate School of Comprehensive Human Sciences and University Hospital, 1-1-1, Tennodai, Tsukuba 305-8575, Japan

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Heitor Rosa, Professor
Department of Gastroenterology and Hepatology, Federal University School of Medicine, Rua 126 n.21, Goiania - GO 74093-080, Brazil

Atif Iqbal, MD
Department of Surgery, Creighton University, c/o Dr Charles J Filipi,Suite 3728, 601 N 30th Street, Creighton University School of Medicine, Omaha NE 68131, United States

Hidekazu Suzuki, Assistant Professor
Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Marco Romano, MD, Professor
Dipartimento di Internistica Clinica e Sperimentale-Gastroenterologia, II Policlinico, Edificio 3, II piano, Via Pansini 5, 80131 Napoli, Italy

Limas Kupcinskas
Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

Olivier Detry, PhD
Department of Abdominal Surgery and Transplantation, University of Liège, CHU Sart Tilman B35, B-4000 Liège, Belgium

oshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman
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Jin-Hong Kim, Professor
Department of Gastroenterology, Ajou University Hospital, San 5, Wonchon-dong, Yeongtong-gu, Suwon 442-721, South Korea

Damian Casadesus Rodriguez, MD, PhD
Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Martin Anlauf, MD
Department of Pathology, University of Kiel, Michaelisstrasse 11, 24105 Kiel, Germany

Dino Vaira, Professor
Department of Internal Medicine and Gastroent, University of Bologna, S.Orsola-Malpighi Hospital - Nuove Patologie, Pad. 5 - via Massarenti 9, Bologna 40138, Italy

Valerio Nobili, PhD
Liver Unit, Research Institute, Bambino Gesù Children's Hospital, S. Onofrio 4 Square, 00165 Rome, Italy

Satoshi Osawa, MD
First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, 431-3192, Japan

Yogesh K Chawla, PhD, Professor
Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

James Neuberger, Professor
Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

Marc Basson, MD, PhD, MBA
Chief of Surgery, John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Otto Schiueh-Tzang Lin, MD
C3-Gas, Gastroenterology Section, Virginia Mason Medical Center, 1100 Ninth Avenue, Seattle, WA 98101, United States

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Department of Surgery, University of Regensburg Medical Centre, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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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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EDITORIAL

Emerging role of IL-23/IL-17 axis in *H pylori*-associated pathology

Roberta Caruso, Francesco Pallone, Giovanni Monteleone

Roberta Caruso, Francesco Pallone, Giovanni Monteleone, Department of Internal Medicine & Centre of Excellence for Genomic Risk Assessment in Multifactorial and Complex Diseases, University "Tor Vergata" of Rome, Rome, Italy
Correspondence to: Giovanni Monteleone, Cattedra di Gastroenterologia, Dipartimento di Medicina Interna, Università Tor Vergata, Via Montpellier, 1, Rome 00133, Italy. gi.monteleone@med.uniroma2.it
Telephone: +39-6-72596158 Fax: +39-6-72596391
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Abstract

Colonization of stomach by *H pylori* is followed by a marked infiltration of the mucosa with polymorphonuclear leukocytes, macrophages, and lymphocytes that very often remains asymptomatic, but in some circumstances can lead to the development of gastroduodenal ulceration, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma. The molecular mechanisms by which *H pylori* triggers and maintains the local immune response are complex, but there is evidence that cytokines produced by both immune and non-immune cells contribute to amplify the ongoing inflammation. *H pylori* infection is associated with a marked mucosal induction of T helper (Th) type 1 and Th17-type cytokines that is governed by specific antigen-presenting cell-derived molecules, such as interleukin (IL)-12 and IL-23. In this paper, we will review the available data on the expression and role of IL-23 and IL-17 in *H pylori*-related gastritis.

Key words: IL-23; IL-17; *H pylori*

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INTRODUCTION

H pylori is a spiral-shaped Gram-negative flagellate bacterium that colonizes the human gastric mucosa and chronically infects more than half of the human population. Infection is inversely correlated with socioeconomic conditions. Most new *H pylori* infections occur in children, but the lack of specific *H pylori*-related clinical signs makes difficult to define the mode of transmission[1]. *H pylori* survives within the gastric mucus layer despite the acidic microenvironment, that limits the growth of most bacteria. This primarily relies upon the ability of *H pylori* to secrete a large amount of urease that breaks down urea into carbon dioxide and ammonia, the latter buffering its environment. Most *H pylori* organisms remain in the mucus layer, even though a small proportion adheres to the mucosal epithelial cells and rarely invades the mucosa[2]. Moreover, *H pylori* can inject into the epithelial cells bacterial products that modify epithelial cell functions[3].

IL-17 IS OVER-PRODUCED IN *H pylori*-COLONIZED GASTRIC MUCOSA

H pylori infection causes a marked infiltration of the gastric mucosa with neutrophils, macrophages, and lymphocytes. Most *H pylori*-infected patients are asymptomatic, but *H pylori*-driven gastritis can lead to the development of gastroduodenal ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma[4]. The level of inflammation increases the risk of disease, but it does not seem to influence which disease develops. In contrast, this is thought to be largely influenced by the pattern of gastric inflammation. In particular, antral gastritis is associated with increased stimulated acid production and predisposes to duodenal ulceration, while corpus-predominant or pangastritis is associated with reduced acid production and predisposes to gastric ulcer and gastric adenocarcinoma[5]. There is also evidence that the degree of gastric infiltration by neutrophils correlates with the development of gastroduodenal ulcerations, and this is in part dependent on the release of damaging inflammatory mediators such as reactive oxygen species[6,7]. Because neutrophils are short-lived, they must be constantly recruited into the infected mucosa from circulation. Antigens released by *H pylori* can stimulate endothelial cells, macrophages and epithelial cells to make huge amounts of chemokines, such as interleukin (IL)-8 and growth-regulated oncogene-alpha, that produce a chemotactic gradient for the migration of neutrophils into the gastric mucosa[8-11]. It is also known that infections with specific *H pylori* strains that possess the cag pathogenicity island (*cag*+) induce significantly higher levels of chemokines than do cag-strains[12]. Both macrophages and epithelial cells also synthesize neutrophil-recruiting chemokines in response to lamina propria mononuclear cell

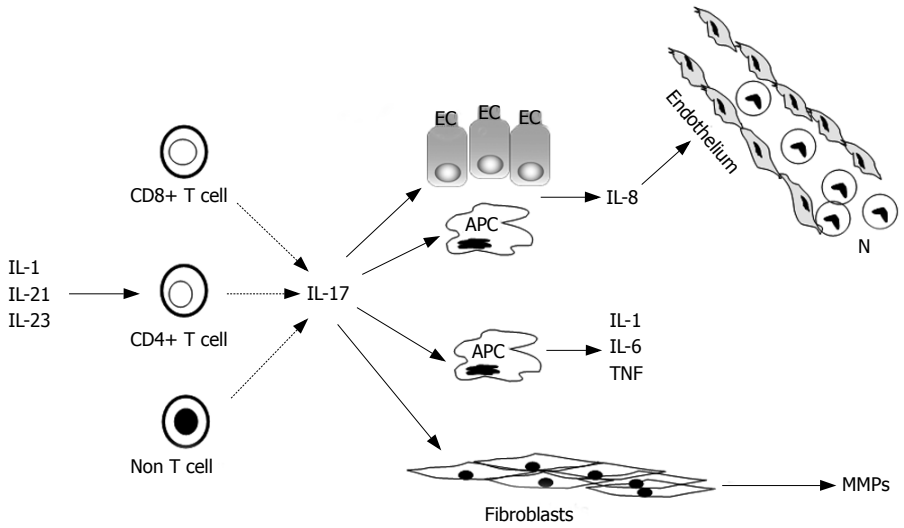

Figure 1 The figure illustrates some of the putative functions of IL-17 in the human gastric mucosa. During *H pylori* infection, IL-17 is produced by both lamina propria (LP) T (CD4+ and CD8+) and non-T cells, through a process that could be positively regulated by IL-1, IL-21, and IL-23. IL-17 stimulates both epithelial cells (EC) and LP antigen presenting cells (APC) to make IL-8, thereby enhancing the recruitment of blood neutrophils (N) into the mucosa. Additionally, IL-17 increases the production of inflammatory cytokines, such as IL-1, IL-6, and TNF by LP APC, as well as it stimulates fibroblasts to secrete matrix metalloproteinases (MMPs), a family of proteases that can cause mucosal degradation.

(LPMC)-derived molecules. In this context, we and others have recently shown that IL-17, a key regulator of neutrophil chemotaxis, is produced in excess in *H pylori*-infected stomach[13-15]. By real-time PCR and Western blotting it was shown that IL-17 up-regulation occurs at both RNA and protein levels in *H pylori*-infected biopsies in comparison to uninfected biopsies either with or without gastritis[13,14]. Notably, among *H pylori*-positive patients, the gastric mucosa at the site of ulcers contains more IL-17 than the non-ulcerated mucosa of the antrum[15]. Several observations suggest that IL-17 plays a decisive role in the neutrophil recruitment to the *H pylori*-infected gastric mucosa. First, IL-17 levels correlate with the number of neutrophils infiltrating the Hp-infected mucosa[15]. Second, both gastric LPMC and epithelial cells express IL-17 receptors and are functionally capable of responding to IL-17 by secreting IL-8[14-16]. Consistently, conditioned media of gastric epithelial cells stimulated with IL-17 enhance the migration of peripheral blood neutrophils, and this effect is inhibitable by a blocking anti-IL-8, but not anti-IL-17 antibody (Figure 1)[14]. Functional analysis of intracellular pathways involved in the induction of IL-8 synthesis by IL-17 revealed that IL-17 activates ERK1/2 MAP kinases in gastric epithelial cells, and that pharmacologic blockade of this pathway significantly inhibits IL-8 secretion[16]. These findings are in line with the demonstration that activated ERK1/2 and IL-8 are more pronounced in gastric epithelial cells isolated from *H pylori*-infected biopsies in comparison to uninfected controls, and that neutralization of endogenous IL-17 in *ex vivo* cultures of *H pylori*-infected gastric biopsies down-regulates the expression of activated ERK1/2 and IL-8[16]. Finally, IL-17 expression positively correlates with IL-8 content in *H pylori*-colonized biopsies[15].

Besides its effects on IL-8 synthesis, IL-17 exerts additional immune-regulatory functions which could influence the magnitude and/or severity of *H pylori*-related gastritis. For example, IL-17 stimulates the production of IL-1, IL-6, and TNF-α by both immune and non-immune cells[17], and induces fibroblasts to make matrix metalloproteinases (MMPs)[18]. MMPs are a family of proteases that can cleave multiple components of the extracellular matrix, thereby contributing to the mucosal damage[19] (Figure 1).

IL-23 CONTROLS IL-17 PRODUCTION IN THE HUMAN GASTRIC MUCOSA

IL-17 was originally named cytotoxic T lymphocyte-associated-8 (CTLA-8), subsequently IL-17, and more recently IL-17A, since it is one of six related members belonging to the IL-17 family (IL-17A-F)[20]. IL-17 was initially described at the message level as a product of human blood activated CD4+ memory T cells. Subsequent studies have shown that IL-17 can be also made by activated CD8+ T cells, TCR$\gamma\delta$+ T cells, and neutrophils[20]. More recently, it was shown that IL-17 is produced by a specific subset of CD4+ T cells, termed T helper (Th) 17-cells, that are distinct from, and antagonized by the classical Th1 or Th2 cells[21]. Th17-cells produce also, but to a lesser extent, TNF-α, IL-6, IL-17F, IL-22, and granulocyte macrophage-colony stimulating factor[22,23]. Flow-cytometry analysis of IL-17 production in gastric LPMC isolated from biopsies of *H pylori*-infected patients showed that CD4+ T cells are a major source of IL-17, even though CD8+ T cells and CD3-negative cells were also positive for IL-17 (Figure 1)[13]. The molecular pathways governing the development of Th17-cells in humans have not been yet elucidated, but studies in murine systems indicate that Th-17 cell differentiation is driven by IL-6 and TGF-β1[24,25]. There is also evidence that expansion and survival of Th17-cells require additional factors, such as IL-23[25]. IL-23 is a heterodimeric protein that is composed by the p40 subunit of IL-12 and a specific subunit, termed IL-23/p19. The functional IL-23 heterodimer is produced by activated dendritic cells (DC), monocytes and macrophages[26]. We have recently shown that IL-23 protein is produced in excess in *H pylori*-colonized mucosa. RNA transcripts for both p40 and p19 subunits were also up-regulated in biopsies from *H pylori*-infected patients, indicating that IL-23 is regulated at the transcriptional level in this condition[13]. These results confirm and expand on data of previous studies showing that *H pylori* enhances IL-23 secretion by monocyte-derived DC[27], and that *H pylori* neutrophil-activating protein (*H pylori*-NAP), a member of a broad super-family of ferritin-like proteins, induces IL-23 production by neutrophils and monocytes[28,29]. Functional studies also revealed that

IL-23 enhances IL-17 synthesis by normal gastric LPMC, and that blockade of endogenous IL-23 activity in cultures of LPMC isolated from *H pylori*-infected biopsies down-regulates IL-17 production[13]. The exact molecular mechanism by which IL-23 regulates IL-17 in *H pylori*-infected mucosa remains to be ascertained. Notably, neutralization of endogenous IL-23 by a blocking anti-IL-23/p19 antibody in cultures of LPMC isolated from *H pylori*-infected biopsies attenuates the expression of active Stat3. Moreover, in normal gastric LPMC, exogenous IL-23 enhances the activation of Stat3, and pharmacologic inhibition of Stat3 suppresses IL-17 production induced by IL-23[13]. Taken together, these results suggest that Stat3 plays a key role in the IL-23-driven IL-17 production during *H pylori* infection. This well fits with the demonstration that Stat3 is essential for the induction and expansion of IL-17-producing cells in response to cytokine stimulation both in vitro and *in vivo*[30]. Such an effect could rely on the ability of Stat3 to bind the promoter of IL-17 gene and enhance its transcriptional activity[31], and/or favor the induction of RORγt, a master regulator of Th17-cell differentiation[32], and the expression of IL-23R.

IL-17 synthesis may be regulated by additional cytokines other than IL-23. IL-1R1-deficient mice fail to mount a robust Th17 response, and IL-1R1-deficient cells do not produce IL-17 in response to IL-23[33]. Since *H pylori* infection enhances the production of IL-1 at the gastric level[34], it is tempting to speculate that this cytokine may act in concert with IL-23 in enhancing IL-17. IL-17 synthesis is also increased by IL-15 in cultures of human and murine CD4+ T cells[35]. However, the fact that IL-15 expression is down-regulated in *H pylori*-infected biopsies argues against a role for IL-15 in the control of IL-17 production during *H pylori*-related gastritis[36]. Th17 cell differentiation is also enhanced by IL-21[37,38], a T-cell derived cytokine that is produced in excess in *H pylori*-colonized stomach[39].

THE ROLE OF IL-23 IN AMPLIFYING *H pylori*-DRIVEN TH1-IMMUNE RESPONSE

During *H pylori* infection, there is a pronounced specific acquired immune response, characterized by generation of antibodies, and differentiation and activation of effector T cells. Although this later includes both a Th1 and a Th2 component, mucosal cytokine profiles imply Th1 predominance[40], and the number of cells producing interferon (IFN)-γ, the key Th1 cytokine, in the *H pylori*-infected human gastric mucosa correlates with the severity of gastritis[41]. Animal models also suggest that the extent of Th1 differentiation is important in pathogenesis. Mice with a predominant Th1 response develop more gastric inflammation during *H pylori* colonization than those with a Th2 response[42-43]. Gastric inflammation and atrophic changes are abrogated in the absence of IFN-γ[44], while IFN-γ infusion into mice, even in the absence of *H pylori* infection, induces pre-cancerous gastric atrophy, metaplasia and dysplasia[45]. IL-12-deficient mice have also reduced gastric inflammatory infiltration and are unable to clear *H pylori* infection[46].

Several virulence factors are reported to promote Th1 responses, including the plasticity region locus jhp0947-jhp0949 which is associated with duodenal ulcer disease[47] and the *H pylori*-NAP[29]. The Th1/Th2 balance is also influenced by phase-variable expression of Lewis blood-group antigens and genomic DNA recombination[48,49].

IL-12 is supposed to be one of the major Th1-inducing factors in *H pylori*-colonized gastric mucosa[50], even though IL-23 may contribute to expand the ongoing Th1 cell response[26]. Indeed, blockade of endogenous IL-23 by anti-IL23/p19 in cultures of LPMC isolated from *H pylori*-colonized biopsies reduces IFN-γ secretion, and stimulation of normal gastric LPMC with IL-23 enhances IFN-γ production. These data are in accordance with the demonstration that IL-23 activates Stat4 and enhances IFN-γ production in cultures of human and murine memory T cells[26], and that in two models of *H. hepaticus*-triggered T cell-dependent colitis, IL-23 enhances both IFN-γ and IL-17 responses that together synergize to trigger severe intestinal inflammation[51,52].

IL-23 and gastric cancer

As pointed out above, *H pylori* is a major factor in the induction of gastritis and its progression to pre-neoplastic lesions and non-cardia gastric cancer. Despite the high prevalence of *H pylori* infection, the risk of gastric cancer in *H pylori*-infected patients is, however, estimated to be approximately 1%-3%. This indicates that infection per se is not sufficient to induce the progression to gastric neoplasia and that additional bacterial and host factors are required[2]. A detailed description of such factors is beyond the scope of this review. In this context it is, however, noteworthy that accumulating evidence would seem to suggest that IL-12 and IL-23 are important mediators in the process that links *H pylori* infection to gastric cancer. Indeed, polymorphisms of IL-12p40 and p35 genes enhance the risk of non-cardia gastric cancers in *H pylori*-infected patients[53]. Moreover, high levels of IL-23 have been documented in human gastric cancers[53]. Nonetheless, no functional study has so far mechanistically linked the activity of IL-12 and IL-23 to gastric cancer. Additionally, studies in murine models of epithelial cancers have shown that IL-23, but not IL-12, is essential for sustaining the tumor-promoting inflammatory process and counteracting the ability of cytotoxic CD8+ T cells to infiltrate tumors[54].

CONCLUSIONS

Although bacterial virulence factors are important in conditioning the outcome of the *H pylori*-driven infection, it is the host attempt to clear the bacterium that causes an exaggerated and inappropriately counter-regulated immune response that may eventually cause tissue damage. Emerging experimental evidence suggests that IL-23/IL-17 pathway is an important driving force the ongoing gastric inflammation in *H pylori*-infected patients. However, further studies will be required to establish the exact contribution of each of these cytokines in the *H pylori*-associated gastric pathology. The availability of strains of mice deficient either for IL-23 subunits or IL-17 should provide valuable models to specifically address these issues.

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REVIEW

Acute renal dysfunction in liver diseases

Alex P Betrosian, Banwari Agarwal, Emmanuel E Douzinas

Alex P Betrosian, Emmanuel E Douzinas, Third Department of Critical Care, Athens University, Evgenidion Hospital, Papadiamantopoulou 20, Athens 11528, Greece
Banwari Agarwal, Intensive Therapy Unit, Royal Free Hospital, London, United Kingdom
Correspondence to: Alex Betrosian, MD, Third Department of Critical Care, Athens University, Evgenidion Hospital, Papadiamantopoulou 20, Athens 11528, Greece. abetrosian@gmail.com
Telephone: +30-210-7208116
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Abstract

Renal dysfunction is common in liver diseases, either as part of multiorgan involvement in acute illness or secondary to advanced liver disease. The presence of renal impairment in both groups is a poor prognostic indicator. Renal failure is often multifactorial and can present as pre-renal or intrinsic renal dysfunction. Obstructive or post renal dysfunction only rarely complicates liver disease. Hepatorenal syndrome (HRS) is a unique form of renal failure associated with advanced liver disease or cirrhosis, and is characterized by functional renal impairment without significant changes in renal histology. Irrespective of the type of renal failure, renal hypoperfusion is the central pathogenetic mechanism, due either to reduced perfusion pressure or increased renal vascular resistance. Volume expansion, avoidance of precipitating factors and treatment of underlying liver disease constitute the mainstay of therapy to prevent and reverse renal impairment. Splanchnic vasoconstrictor agents, such as terlipressin, along with volume expansion, and early placement of transjugular intrahepatic portosystemic shunt (TIPS) may be effective in improving renal function in HRS. Continuous renal replacement therapy (CRRT) and molecular absorbent recirculating system (MARS) in selected patients may be life saving while awaiting liver transplantation.

Key words: Hepatorenal syndrome; Transjugular intrahepatic portosystemic shunt; Continuous renal replacement therapy; Molecular absorbent recirculating system; Acute liver failure; Systemic vascular resistance; Renal blood flow

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INTRODUCTION

Renal and liver dysfunction often present together, either as part of multiorgan failure in a critically ill patient, or as a result of failure of each organ independently. Three major clinical scenarios can be identified in which liver and renal dysfunction coexist; diseases simultaneously involving the liver and the kidney, or a primary hepatic disorder with secondary renal dysfunction, or vice versa[1]. Concomitant renal and liver dysfunction may share common pathogenetic mechanisms. Renal dysfunction in this setting usually develops gradually, with the exception of certain infections such as leptospirosis, some viral hemorrhagic fevers and toxin-mediated injuries such as acetaminophen poisoning, which cause acute insufficiency of both organs[2]. Renal failure secondary to liver dysfunction is generally functional in nature and occurs in the absence of significant alterations in renal histology (pre-renal). However, intrinsic renal abnormalities can also complicate acute or chronic liver disease (intrinsic renal failure)[3]. Obstructive uropathy that leads to postrenal acute renal failure only rarely develops in chronic liver disease (papillary necrosis in alcoholic liver disease, bleeding in the urinary tract due to severe coagulopathy)[4]. Hepatorenal syndrome (HRS) is a unique form of functional renal failure (pre-renal) that often complicates advanced liver disease, hepatic failure or portal hypertension[5].

EPIDEMIOLOGY

The incidence of renal failure in acute liver failure (ALF) varies from 40% to 85%, depending on the etiology; paracetamol poisoning leads to renal failure in up to 75% of patients[6]. Renal failure following paracetamol overdose may also occur in the absence of ALF, and has a good prognosis[7]. In non-paracetamol cases the incidence of renal failure is usually accompanied by worsening encephalopathy and is associated with a poor outcome[5,6].

Acute renal failure (ARF) in patients with cirrhosis, particularly with advanced liver disease, seems to be common; however, the exact incidence is unknown and is probably underestimated[3]. This may be explained by the fact that patients with cirrhosis tend to have falsely low serum creatinine levels due to decreased hepatic creatinine synthesis and decreased skeletal muscle mass[8]. ARF in patients with cirrhosis frequently accompanies complications such as bacterial peritonitis or other

sepsis, hypovolemia from gastrointestinal bleeding or excessive diuretic therapy, administration of nephrotoxic drugs/contrast agents, or development of HRS[2,3]. The probability of the occurrence of HRS in patients with cirrhosis and ascites at 1 and 5 years is 18% and 39%, respectively, with mortality approaching 100% in type I HRS without specific therapy. The median survival time in these patients without liver transplantation was only 12 d after diagnosis in one study[9]. However, this seems to have improved with terlipressin and albumin therapy[10]. The development of ARF in patients with cirrhosis has significant prognostic importance. In patients with cirrhosis admitted to hospital with acute upper gastrointestinal hemorrhage, development of ARF forms an independent predictive factor for death[11,12].

PATHOPHYSIOLOGY OF RENAL FAILURE IN LIVER DISEASE

The mechanism underlying the development of ARF in advanced liver disease and cirrhosis is complex and includes interactions between changes in the systemic arterial circulation, portal hypertension, activation of vasoconstrictors and suppression of vasodilatory factors acting on the renal circulation[1,3,13]. The pathophysiology of functional renal failure in ALF is similar to that in cirrhosis[6,14], and patients with ALF may develop portal hypertension, but to a lesser degree than in those with cirrhosis[6]. The common pathway of renal dysfunction is the development of intense systemic arterial vasodilation, which follows increased release of endogenous vasodilators, especially nitric oxide, which escapes from the splanchnic to the systemic circulation through portosystemic shunts[1,13-15]. The systemic vasodilation leads to a reduction in systemic vascular resistance (SVR) and consequent high cardiac output and hyperdynamic circulation. However, the increase in cardiac output may be inadequate to compensate for the drop in SVR, especially in ALF, which results in hypotension with mean arterial pressure (MAP) commonly falling to 60-70 mmHg, which is on the pressure-dependent part of the autoregulatory curve of renal blood flow (RBF)[6,16]. In healthy individuals, autoregulation of RBF occurs until the renal perfusion pressure falls below 60-70 mmHg. Altered renal vascular autoregulation, as seen in sepsis, may also be present in the hyperdynamic circulatory failure of ALF, making RBF directly dependent on blood pressure[17]. In some patients with cirrhosis, especially alcoholics, the presence of cardiomyopathy and heart failure may further render them susceptible to renal compromise secondary to hypoperfusion[18].

The normal homeostatic response to vasodilation is activation of several neurohumoral mechanisms, such as the renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system (SNS), and arginine-vasopressin (AVP) which leads to intense vasoconstriction and salt and water retention, in an attempt to maintain blood pressure and perfusion of vital organs[2,5,14,17]. Other vasoconstrictors, such as eicosanoids, endothelins, thromboxane A2 and leukotrienes may further exacerbate this[6,13,14,19].

RBF is kept within normal limits in the early stage of the liver disease, due to the release of certain local vasodilators such as prostaglandins. However, in situations in which circulatory volume is acutely diminished, as in gastrointestinal hemorrhage, renal hypoperfusion and subsequent pre-renal azotemia may occur. As the liver disease progresses, there is extreme vasoconstriction of the renal vascular bed that predisposes the kidneys to development of HRS[2,20]. The presence of tense ascites may further impair renal perfusion. The continuing vasoconstriction and raised vascular resistance results in contraction of the mesangium, with a reduction in glomerular surface area, which leads to acute tubular necrosis (ATN)[21].

CAUSES OF ACUTE RENAL FAILURE IN LIVER DISEASE

Pre-renal

Patients with advanced liver disease are susceptible to pre-renal azotemia, secondary to the development of relative hypovolemia and reduced effective central blood volume. The initial event is development of portal hypertension, which then leads to splanchnic and systemic vasodilatation mediated by NO and other vasodilators. Vasodilatation seems to be the main mechanism, however, underfilling has also been suggested, which is explained by fluid sequestration in the peritoneal cavity[22-24]. True hypovolemia can further exacerbate renal dysfunction in these patients. It can be induced by gastrointestinal tract hemorrhage from varices, peptic ulcers, gastropathy or other sources, excessive diuresis, vomiting and diarrhea, or can be aggravated by large volume paracentesis without intravascular volume replacement[3,11,13]. Bacterial infections and the use of nonsteroidal anti-inflammatory drugs can also precipitate pre-renal azotemia in these patients[3,25]. Patients with ALF and cirrhosis are abnormally susceptible to infection. Hemodynamic abnormalities induced by cytokines and vasodilating substances, such as NO in spontaneous bacterial peritonitis, play an important role in the pathogenesis of renal dysfunction. Additionally, development of septic shock further impairs renal function[25,26].

Intrinsic renal

Intrinsic renal disease can either complicate acute liver diseases as a result of exposure to certain drugs, toxins and infections, or be a part of chronic liver disease. The former usually is tubular interstitial in nature and presents as ARF, while the latter predominantly leads to glomerulopathy and is characterized by stable kidney disease. The causes of intrinsic renal involvement in liver diseases are numerous and are beyond the scope of this review. This review will mainly concentrate on acute tubular necrosis (ATN) and HRS, with other causes listed in the Table 1.

ATN

Direct cellular toxicity with ATN and hepatocyte necrosis have been observed in paracetamol intoxication[16]. Despite the nephrotoxic potential of this drug, functional renal failure is also seen secondary to ALF. Renal failure rarely

occurs in the absence of liver failure[7]. Depletion of glutathione is believed to be the cause of both ARF and ALF[36]. Aspirin is another analgesic drug that can cause dose-related liver damage and renal failure in susceptible patients. Of special interest is the association of aspirin, used to treat symptoms of influenza or varicella, with Reye's syndrome[27,37]. The mechanism of renal damage is due to cyclooxygenase inhibition thereby preventing the production of vasodilatory prostaglandins.

ATN with acute renal insufficiency in patients with stable liver disease often follows insults such as hypovolemic shock, major surgical procedures and use of nephrotoxic drugs or contrast agents, infection or sepsis. The functional renal abnormalities associated with advanced liver disease and cirrhosis increase the susceptibility of the kidneys to the development of ATN. These renal abnormalities may either be ischemic or toxic in origin. The mechanism of renal failure is similar in both, and results from a reduction in glomerular filtration rate (GFR) due to impaired glomerular capillary pressure, disrupted integrity of tubular epithelium, and tubular obstruction from casts composed of detached epithelial cells, cellular debris and pigments (hemoglobin and myoglobin)[38]. It should be noted that all causes of pre-renal azotemia might lead to ischemic tubular injury if left untreated[21,39].

The association of obstructive jaundice and ARF is well-established. Susceptibility to renal failure in obstructive jaundice is a combination of cardiovascular instability due to defective vascular reactivity and blunted myocardial contractile response as a result of the deleterious effects of bile constituents. Furthermore, the natriuretic effects of bile acids can cause volume depletion and exaggerate the effective arterial underfilling. However, there is a direct nephrotoxicity of biliary products at very high levels of bilirubin (> 30 mg/dL) in both children and adults[40,41].

Contrast medium is a well-known precipitant of renal failure in hospitalized patients, particularly in the presence of predisposing conditions such as reduced effective blood volume, dehydration and diabetes mellitus. Cirrhosis has been considered a potential predisposing factor. However, a prospective study in euvolemic patients with cirrhosis has shown that administration of contrast medium is not associated with adverse effects on renal function, which suggests that cirrhosis per se should not be considered a risk factor for contrast media nephrotoxicity[42,43]. However, it may play a role in septic cirrhosis, or in patients who have bleeding or who have undergone transarterial embolization for hepatocellular carcinoma.

Table 1 Intrinsic kidney involvement in liver diseases

Tubulo-interstitial involvement
1 Drugs[7] (paracetamol, aspirin, carbon tetrachloride, halogenated hydrocarbons, immunosuppressant agents)
2 Toxins[28-35] (Galerina family of mushrooms, hemoglobin, myoglobin, bilirubin, contrast agents)
3 Infections (leptospirosis, malaria, hepatitis)
4 Hypersensitivity reactions (sulphonamides, salicylates, *etc.*)
Glomerular involvement
1 Drugs[7] (carbon tetrachloride)
2 Hepatitis[28-35] A, B, C
3 Type Ⅱ mixed cryoglobulinemia[28-35]
4 IgA nephropathy[28-35] (alcoholic cirrhosis, HCV cirrhosis)
5 Others (sickle cell disease, hemochromatosis, acute fatty liver and toxemia of pregnancy)
Vascular
1 Vasculitis
2 Toxemia of pregnancy and HELLP syndrome

HRS

HRS is a unique form of functional renal failure that often complicates advanced liver disease, hepatic failure or portal hypertension[3,9,20]. The incidence of HRS in patients with cirrhosis hospitalized for ascites is about 10%. The syndrome is characterized by intense intrarenal vasoconstriction in the presence of vasodilation of systemic and splanchnic circulation, which triggers a reduction in peripheral vascular resistance and a decrease in effective systemic circulatory volume, despite an overall expanded total extracellular fluid volume. The majority of patients have clinical evidence of advanced cirrhosis[20]. However, HRS may occur in patients with fulminant viral and alcoholic hepatitis[36]. Two patterns of HRS can be identified.

Type 1 HRS is characterized by a rapidly progressive reduction of renal function, defined as either doubling of the initial serum creatinine to > 2.5 mg/dL or a 50% reduction in GFR to < 20 mL/min over a 2-wk period. Precipitating factors include spontaneous bacterial peritonitis (SBP), major surgical procedures, and acute alcoholic hepatitis. It follows a fulminant course with development of oliguria, encephalopathy, and marked hyperbilirubinemia, and is associated with very poor prognosis, with death occurring within 1 mo after presentation[27,36].

Type 2 HRS is characterized by a more benign course, with a stable reduction in GFR over weeks to months, accompanying diuretic-resistant ascites and avid sodium retention[36]. The pathogenesis of HRS is incompletely understood. It may be the result of an imbalance between renal vasodilators and vasoconstrictors, with the latter predominating. This interplay between the intrarenal mechanisms is triggered by one of the above-mentioned precipitating factors, which exacerbate the previously diminished cardiac and renal function[14,20].

The diagnostic criteria of HRS as proposed by the International Ascites Club are listed in Table 2[44]. Only the major criteria are necessary for the diagnosis of HRS, while the minor criteria are supportive. The diagnosis of HRS is one of exclusion and depends mainly on the level of serum creatinine, despite the fact it does not provide an accurate reflection of GFR in patients with cirrhosis[8]. Patients with cirrhosis with serum creatinine > 1.5 mg/dL have a GFR (estimated by inulin clearance) of < 30 mL/min, which represents one quarter of the normal GFR for healthy subjects of the same age[45]. HRS is a form of functional renal failure, therefore, the

Table 2 Diagnostic criteria of HRS

Major criteria
1 Chronic or acute liver disease with advanced liver failure and portal hypertension
2 Low GFR, as indicated by a serum creatinine of > 1.5 mg/dL or a 24-h creatinine clearance < 40 mL/min
3 Exclusion of shock, ongoing bacterial infection, volume depletion, and the use of nephrotoxic drugs
4 No improvement in renal function despite stopping diuretics and volume repletion with 1.5 L of saline
5 No proteinuria or ultrasonographic evidence of obstructive uropathy or parenchymal renal disease

Minor criteria[1]
1 Urine volume lower than 500 mg/day
2. Urine sodium lower than 10 mEq/L
3 Urine osmolality > plasma osmolality
4 Urine blood cells < 50 per high-power field
5 Serum sodium concentration lower than 130 mEq/L

[1]Only major criteria are necessary for the diagnosis of hepatorenal syndrome.

characteristics of urine are those of pre-renal azotemia with oliguria, low sodium concentration, and increased osmolality and urine to plasma ratio. These parameters are not considered essential for the diagnosis of HRS because they may overlap with different types of renal failure[44,45].

DIFFERENTIAL DIAGNOSIS OF ARF IN LIVER DISEASE

The differential diagnosis of ARF in advanced liver disease includes pre-renal failure, intrinsic renal failure and HRS (Table 3). The diagnostic evaluation relies upon clinical and laboratory data, including examination of urinary sediment and urinary chemistry, as well as appropriate ultrasonographic and radiological investigations[3,14,20]. Renal biopsy generally is not necessary for the diagnosis of ARF in liver disease, but is useful in excluding an intrinsic renal disorder[3]. A history of gastrointestinal hemorrhage, vomiting or diarrhea, exposure to nephrotoxic medication, or features suggestive of sepsis may provide important diagnostic information. Arterial hypertension, which is an unexpected finding in patients with cirrhosis, suggests glomerulonephritis[46]. The course of renal response to fluid challenge or vasoconstrictor therapy can also help differentiate causes of acute azotemia in liver disease. Rapid improvement in renal function denotes pre-renal failure, whereas mild or no improvement represents ATN or HRS[39,44]. Vasoconstrictor agents such as terlipressin or noradrenalin can sometimes be used to differentiate HRS and ATN, with improvement of GFR in favor of HRS[47,48]. Urine indices such as osmolality, sodium concentration, urine:plasma osmolality ratio (U/Posm) and urine:plasma creatinine ratio (U/Pcreat), are useful theoretical tools for differential diagnosis of the three principal causes of ARF in liver disease. However, in reality apart from urinary sediments these are often not clear cut.

Duplex Doppler ultrasonography, is a sensitive method of assessing intrarenal hemodynamics in patients with stable cirrhosis and ascites, in whom the renal artery resistive index is significantly increased and correlated with GFR and plasma renin activity[49]. However, this method is only useful in stable cirrhosis and may not be applied to acute situations.

Table 3 Differential diagnosis of ARF in advanced liver disease

	Prerenal failure	Intrinsic renal failure	HRS
Urine sodium	< 10	> 30	< 10
U/Pcreat	> 30:1	< 20:1	> 30:1
U/Posm	UO > PO	UO = PO	UO > PO
Urine sediment	Normal	Casts, cellular debris	Unremarkable
History disease	Profound volume	Volume contraction	Advanced liver disease
Clinical course (renal response)	Contraction	Nephrotoxic agent sepsis	Tence ascites
Fluid challenge	+	-	-
Vasoconstriction	±	-	+
Ultrasound	Elevated resitive index	Elevated resitive index	Elevated resitive index

MANAGEMENT OF ARF IN LIVER DISEASE

Management of ARF in liver disease should follow the same general principles as for the management of renal failure of any etiology, as well as specific measures for the liver disease. Combined ARF and ALF should ideally be managed in an intensive care or high-dependency setting. Initial management comprises correction of life-threatening abnormalities such as hyperkalemia, hypoglycemia, severe blood gas abnormalities, gross fluid overload and coagulation disorders, which may lead to bleeding and worsening renal function[50]. Although bleeding problems in patients with chronic liver failure are much less than previously thought, as a result of normal thrombin generation, renal failure superimposed on liver failure may have a negative impact on bleeding diathesis and worsening of hemorrhage[51].

Potential nephrotoxic drugs should be discontinued if possible, diuretic therapy interrupted, and infusion of crystalloid or colloid solutions commenced, based on clinical assessment and hemodynamic monitoring. In ALF complicated by intracranial hypertension, in addition to general measures to treat cerebral edema, early continuous renal replacement therapy (CRRT) may be considered, especially when patients are oligo-anuric and are taking mannitol[5,6].

In patients with chronic liver disease, management of ARF must focus on pre-renal failure, HRS and ATN. Upper gastrointestinal hemorrhage needs transfusion of plasma expanders and packed red blood cells, while measures are being taken to identify and treat the bleeding focus[12]. Intestinal and renal fluid losses should be replaced with appropriate fluid. Evidence of sepsis should be meticulously sought and a non-nephrotoxic broad-spectrum antibiotic regimen commenced, regardless of the etiology of sepsis. Current literature does not support the role of low-dose dopamine in the prevention and treatment of sepsis-induced renal vasoconstriction and

failure[52]. Vasopressin and terlipressin provide adequate splanchnic vasoconstriction and have been used not only in patients with cirrhosis and HRS, but also in sepsis in resistant cases[53].

Many different therapeutic approaches have been proposed for the management of HRS[2,13,27,38]. Unfortunately, most treatment measures result in only transient beneficial effects on renal function, and are not consistently associated with improvement in patient survival. Liver transplantation remains the definitive treatment for HRS, but is associated with higher hospital mortality compared to those without HRS who are treated with transplantation[54]. Thus, every attempt should be made to prevent this severe complication or reverse it when managing patients with cirrhosis and ascites. In recent years, new treatment strategies such as the use of vasoconstrictor drugs, along with plasma volume expansion, or insertion of TIPS, have shown some promise[20]. These treatments may prolong survival time and, therefore, act as a bridge to liver transplantation in these patients. Vasoconstrictors used for HRS include vasopressin analogues (ornipressin and terlipressin), somatostatin analogues (octreotide), and alpha-adrenergic agonists (midodrine and noradrenalin)[20]. In type 1 HRS terlipressin in combination with albumin has shown to result in greater improvement in renal function compared to terlipressin alone[10,53]. Pharmacological treatment, when combined with interventional techniques, such as transjugular intrahepatic portosystemic shunt (TIPS), may further improve renal function in HRS[27,55]. However, TIPS is frequently associated with significant side effects, particularly hepatic encephalopathy and impairment of liver function, and its role in the management of HRS needs to be established by prospective, controlled investigations[56].

The molecular adsorbent recirculating system (MARS) has been used in the treatment of acute decompensation of chronic liver disease (ADCLF), ALF and HRS[57]. This liver support system utilizes either intermittent (6-8 h daily) or continuous hemodialysis with dialysate enriched with 20% human serum albumin as a means to remove albumin-bound toxins (bilirubin, bile acids, fatty acids, tryptophan, aromatic amino acids, and copper). The first randomized trial of MARS evaluated 13 ADCLF patients with type 1 HRS. Five patients treated with hemodiafiltration alone died within 7 d, whereas three of eight patients treated with MARS were alive at 7 d, and two of eight were alive at 30 d[58]. Another recent randomized control trial, which included 24 patients with ADCLF, showed improvement in hyperbilirubinemia and hepatic encephalopathy and 30-d survival in patients treated with MARS. There was also improvement in renal function in the MARS group[59]. Until now, MARS has shown no benefit in improving survival in acute exacerbations of chronic liver failure. Likewise, MARS in the context of ALF has not been studied in control trials.

CRRT

Theoretically, the indications for CRRT in advanced liver disease and renal failure should be similar to those for general population. However, in view of the underlying disease, renal support should only be provided to those with a clear goal of hepatic management and a potential positive outcome i.e., the possibility of hepatic recovery or liver transplantation[60,61]. Measures such as volume expansion with albumin and the use of terlipressin should be tried before considering RRT. In fulminant hepatic failure (FHF), CRRT has become a major part of the routine management. Although there are no randomized trials to prove its efficacy, it can be assumed that it has contributed in part to the improvement in mortality. The continuous form provides greater hemodynamic, and more importantly, greater intracranial pressure ICP stability than the intermittent forms[62]. Continuous techniques (hemofiltration/hemodiafiltration) are preferred since they are associated with greater cardiovascular stability and allow gradual fluid removal, which can be adapted to actual needs and the infusion volume required for drug therapy and nutritional support[61].

PREVENTION OF RENAL FAILURE IN ADVANCED LIVER DISEASE

Two different strategies can be used to prevent HRS. The first is to perform liver transplantation in patients with cirrhosis and ascites before HRS develops. The identification of factors associated with a high risk of developing HRS and the use of duplex Doppler ultrasonography to assess the renal artery resistive index in the follow-up of these patients may be useful for this purpose[9,24,49]. The second strategy is to prevent the development of renal impairment in patients by avoiding the precipitating factors i.e., prompt management of bleeding and infection. A recent study has indicated that the development of HRS in patients with SBP can be effectively prevented by the addition of albumin to antibiotic (cefotaxime) therapy (1.5 g/kg human albumin intravenously at the time of diagnosis of the infection and 1 g/kg intravenously 48 h later). The proportion of patients who developed HRS and the in-hospital mortality was significantly lower in the cefotaxime-plus-albumin group that in the cefotaxime alone[63]. The beneficial effect of albumin is probably related to its ability to prevent circulatory dysfunction and subsequent activation of vasoconstrictor systems that occur during infection[63].

ARF POST LIVER RESECTION AND LIVER TRANSPLANTATION

Liver transplantation is the optimal treatment for patients with end-stage liver disease and ALF. The immediate outcome of orthotopic liver transplantation (OLT) is dependent on several factors, including pretransplant renal function and hemodynamic conditions in the operative and postoperative periods[63]. The prevalence of renal insufficiency in patients before transplantation varies from 10% to 20%, although many of these patients may have HRS, which is potentially a reversible condition.

Pretransplant renal dysfunction is a poor prognostic marker[64]. The reason for the poorer prognosis of these patients may be related to the persistence of the hyperdynamic circulation after liver transplantation, and to the fact that these patients seem to be more susceptible to damage from immunotherapy with cyclosporine or tacrolimus[65]. Additionally, pretransplant renal failure increases the incidence of postoperative sepsis, the need for pre- and postoperative dialysis, the number of days spend in the intensive care unit, and short-term graft and patient survival rates[64,66]. Renal failure is a frequent complication after OLT. It is usually acute, appears early after transplantation, and has an unfavorable effect on prognosis of liver transplant patients. The reported incidence ranges from 12% to 61%, according to the criteria used for defining ARF (serum creatinine ranging from 1.5 to 3 mg/dL or higher)[66,67]. Post-transplant ARF is usually caused by ATN due to perioperative complications (circulatory instability, duration), sepsis, repeated rejection, calcineurin-mediated renal vasoconstriction or nephrotoxic drugs (e.g., aminoglycosides and amphotericin B).

Prevention of renal failure after liver transplantation is not easy. The benefit of RRT with continuous venovenous hemodialysis before and after liver transplantation has been established[68]. Administration of aprotinin, an antifibrinolytic agent may be of benefit in the prevention of renal failure in adult patients undergoing OLT, by reducing intraoperative blood loss. Despite its potential nephrotoxic side effects, the administration of regular doses of aprotinin does not lead to a higher incidence of renal failure in these patients[69].

Treatment of HRS prior to OLT could also be beneficial for the prevention of ARF[63,66]. Finally, a delay in the introduction of nephrotoxic immunosuppresive drugs could be helpful in the prevention of post-transplant ARF, especially in high-risk patients.

CONCLUSION

Acute renal dysfunction is common in patients with acute and chronic liver disease. The presence of renal failure in this group of patients significantly affects mortality. Several advances have occurred in the management of this complication in the last decade. Improvement in the management of ARF in ALF is reflected in our better understanding of the disease process and better hemodynamic and renal replacement support. In advanced liver disease complicated by liver failure, terlipressin and early formation of TIPS have shown some promise. The role of MARS, bioartificial liver support, high-volume hemofiltration, and therapeutic plasma exchange, are still unclear and need further evaluation. Finally, as liver transplant is the definitive treatment, further education to increase the organ donation pool may be the best way forward.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Novel genetic markers in inflammatory bowel disease

Lorena Rodríguez-Bores, Gabriela C Fonseca, Marco A Villeda, Jesús K Yamamoto-Furusho

Lorena Rodríguez-Bores, Gabriela C Fonseca, Marco A Villeda, Jesús K Yamamoto-Furusho, IBD Clinic, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga 15 colonia Sección XVI, Tlalpan, CP 14000, México
Correspondence to: Jesús K Yamamoto-Furusho, MD, PhD, Head of IBD Clinic, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga 15 colonia Sección XVI, Tlalpan, CP 14000, México. kazuofurusho@hotmail.com
Telephone: +52-55-55733418-2705 Fax: +52-55-56550942
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Abstract

Genetic factors play a significant role in determining inflammatory bowel disease (IBD) susceptibility. Epidemiologic data support genetic contribution to the pathogenesis of IBD, which include familial aggregation, twin studies, racial and ethnic differences in disease prevalence. Linkage studies have identified several susceptibility genes contained in different genomic regions named IBD1 to IBD9. Nucleotide oligomerization domain (NOD2) and human leukocyte antigen (HLA) genes are the most extensively studied genetic regions (IBD1 and IBD3 respectively) in IBD. Mutations of the NOD2 gene are associated with Crohn's disease (CD) and several HLA genes are associated with ulcerative colitis (UC) and CD. Toll like receptors (TLRs) have an important role in the innate immune response against infections by mediating recognition of pathogen-associated microbial patterns. Studying single-nucleotide polymorphisms (SNPs) in molecules involved in bacterial recognition seems to be essential to define genetic backgrounds at risk of IBD. Recently, numerous new genes have been identified to be involved in the genetic susceptibility to IBD: NOD1/Caspase-activation recruitment domains 4 (CARD4), Chemokine ligand 20 (CCL20), IL-11, and IL-18 among others. The characterization of these novel genes potentially will lead to the identification of therapeutic agents and clinical assessment of phenotype and prognosis in patients with IBD.

Key words: Genetic; Inflammatory bowel disease; Human leukocyte antigen; Nucleotide oligomerization domain; Toll like receptors; Susceptibility

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INTRODUCTION

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD) which are characterized by chronic illness of unknown etiology; however, its development is influenced by genetic, environmental and immunological factors[1].

Epidemiological studies suggest that genetic susceptibility is a major contributing factor to IBD. Molecular data from total genome scans and from candidate gene studies have led to the identification of genetic determinants of susceptibility and disease phenotype of UC and CD. The primary goal of genetic research is to identify genetic variants within specific genes which could modify homeostasis and increase disease susceptibility. There is growing attention to the innate immune response and the interaction between genetic factors and bacterial flora, or pathogen-associated molecular patterns in order to understand the contribution of environmental factors to disease susceptibility, as well as the phenotype based on a more precise molecular basis of disease pathogenesis. Clinical impact of the genetic findings has helped in understanding the heterogeneity of IBD in location, age at onset, clinical course and predicting response to conventional treatment.

GENETIC MODEL

Clinical and epidemiological data do not support a simple Mendelian model of inheritance for IBD. In its place CD and UC are considered to be complex polygenic diseases. Two major methods for identification of genes in complex multifactorial diseases are used, the positional cloning method based on association studies and the candidate gene analysis. Linkage analysis allows scanning of the whole genome studying the co-segregation of the disease with a marker within families, constituting an important method where allele sharing between affected sibling pairs is used. An alteration of the observed ratio of sharing contrasting the expected is interpreted as evidence for linkage with a particular marker. On the other hand, the candidate gene analysis attempts to determine the

Table 1 Major regions and genes in IBD

Region	Localization	Involved genes
IBD1	Chromosome 16	NOD2/CARD15, IL-4R, CD11B
IBD2	Chromosome 12	Vitamin D receptor (VDR), STAT6, Interferon γ, β7 integrine.
IBD3	Chromosome 6	Major histocompatibility complex (MHC): Class Ⅰ, Ⅱ, Ⅲ.
IBD4	Chromosome 14	T- Lymphocyte receptor (TCR) and Leukotriene B4
IBD5	Chromosome 5	Organic cations transporter (OCTN), *Drosophila long disc homologue gene 5* (DLG5), *Multidrug resistant gene* (MDR1), IL-6, CD14
IBD6	Chromosome 19	Thromboxane A2, Leukotriene B4, ICAM-1
IBD7	Chromosome 1	Transforming growth factor Beta (TGFβ), TNFα receptors.
IBD8	Chromosome 16	Under research
IBD9	Chromosome 9	CCR-5, CCR9, IL-12

NOD2/CARD15: Nucleotide oligomerization domain 2/Caspase-activation recruitment domains 15; STAT6: signal transducer and activator of transcription-6; ICAM-1: intracellular adhesion molecule 1; CCR5: CC-chemokine receptor5.

importance of specific genes in disease pathogenesis, using case-control cohorts or trios of affected progeny with both parents. The allelic frequencies or the transmission of a single-nucleotide polymorphism (SNP) towards affected progeny is studied and the differences between patients and controls might point towards the implication of a particular gene in the pathogenesis of the disease under investigation. It also includes positional candidate genes which are found in areas of linkage defined by genome screening. As CD and UC are likely to share some susceptibility genes, it has been proposed a genetic model of UC and CD where the two are polygenic disorders, sharing some susceptibility *loci*, but differing in others[2]. Linkage studies support this concept because some *loci* appear to interfere with susceptibility to IBD, which have also been implicated in the abnormal immune response and those susceptibility genes could interfere with the disease phenotype such as extension, need for procto-colectomy, extraintestinal manifestations, as well as the response to different treatments. From most of the genome-wide scans performed in IBD, a number of susceptibility regions on several chromosomes have been found[3-6] and according to their initial date of reporting and independent confirmations, the regions on such chromosomes have been renamed as IBD1-9 (Table 1).

IBD1

Accurate mapping of the IBD1 locus has led to identification of the underlying gene called the NOD2/Caspase-activation recruitment domains 15 (CARD15) gene located on the pericentromeric region on the long arm of chromosome 16 (16p12.3) extending to 16q13[7-10]. Several studies have demonstrated the identification of NOD2/CARD15 gene within the IBD1 locus as a susceptibility gene in CD, suggesting that approximately 25%-30% of the genetic susceptibility in CD can be explained by mutations in NOD2/CARD5 though most of these studies have shown no association between NOD2 mutations and susceptibility to UC[11,12]. Thirty non-conservative polymorphisms have been identified within the gene that are associated with CD and only three are common (Arg702Trp, Gly908Arg and Leu1007insC). The three common variants, however, account for approximately 82% of the mutated alleles[13]. Nevertheless, these mutations seem to have different effects on the risk of developing CD: Arg702Trp, Gly908Arg and a deletion in the last 33 aminoacids Leu1007finsC, which are present in 43% of the patients with CD (10%-30% is heterozygous and 2%-15% is homozygous for these mutations)[8-10]. These variants in NOD2 have been associated with certain clinical features of CD[14]. The NOD2 contribution seems to be stronger in Ashkenazi Jewish population who has a higher frequency of the Gly908Arg NOD2 variant. In relation to phenotypic expression and translation into the clinic, some associations between NOD2 mutations and earlier-onset disease in adult populations, fistulizing disease, fibrostenosing disease behavior and increased risk of need for surgery in children have been described[15-18].

CARD gene codes for a protein expressed in several cells of innate immunity, epithelial cells and Paneth cells[11,12]. This protein consists of two N-terminal CARD, a central nucleotide-binding domain and a C-terminal leucin-rich-repeat region (LRR). It has been reported that CARD15 is implicated in the recognition of a bacterial product peptidoglycan-derived muramyl dipeptide (MDP) that enters into the cytosol via a transporter protein hPepT1 and interacts with the LRR of NOD2. Mutations within the leucine-rich region are associated with CD, as mutations within the nucleotide-binding domain are associated with granulomatous diseases[19]. Through the recognition of MDP, secretion of alpha-defensins is stimulated for protection against microbial invasion. In CD patients, a diminished expression of alpha-defensins has been found in those carriers with NOD2 mutation[20,21]. It is known that through this recognition of bacterial products the nuclear factor kappa B (NFκB) activation is regulated[10,22].

Evidence show that NOD2 protein acts as an important regulator of NFκB activation in response to the Toll-like receptor (TLR) 2 activation system leading to its down regulation[23,24]. However in carriers of mutant protein, this process does not occur and proinflammatory cytokines are produced with a Th1 profile[25,26].

IBD2

This region is located on chromosome 12 showing greater linkage evidence in UC compared with CD. A number of possible candidate genes have been investigated including signal transducer and activator of transcription-6 (STAT6), INFγ, metalloproteinase (MMP18), Vitamin D receptor (VDR) and β7 integrin family that could be associated with the susceptibility to IBD. Parkes *et al*[27] found that IBD2 appears to make a major contribution to UC susceptibility but has only a relatively minor effect on CD.

Vitamin D receptor

The Vitamin D receptor (VDR) is a member of a steroid receptor family and mediates the effects of the active metabolite 1.25 $(OH)_2$ vitamin D_3 by regulating

transcription of a number of different genes. It is synthesized by activated macrophages. It is expressed by monocytes and activated B and T lymphocytes. It activates monocytes and macrophages but suppresses lymphocyte proliferation and immunoglobulin production, and also inhibits transcription factor NFκB, and the production of IL-2, IL-12 and interferon γ. 1.25 $(OH)_2D_3$ is the form of vitamin D that binds to the VDR and inhibits experimental autoimmunity[28,29]. Vitamin D deficiency and VDR deficiency have been shown to exacerbate chronic IBD in IL-10 knock out mice[29,30]. Absence of the VDR results in mice that are extremely susceptible to chemical injury in the gut[30]. The linked SNPs found at the 3' end of VDR are: *Bsm*I, *Apa*I, *Taq*I, and the exon 2 splice site *Fok*I polymorphism[31,32]. *Fok*I polymorphism has been associated with osteoporosis, *Taq*I polymorphism with the risk of prostate cancer[33] and recently homozygotes for the *Taq*I allele have been shown to have altered susceptibility to a variety of infectious diseases[34]. Simmons *et al*[35] studied 403 European Caucasian patients with IBD, and found significantly more homozygotes for the *Taq*I polymorphism among patients with CD than in patients with UC or controls, providing evidence for a genetic association between CD susceptibility and a gene that lies within one of the candidate regions determined by linkage analysis. Dressner-Pollak *et al*[36] found that *Bsm*I VDR gene polymorphism is associated with increased susceptibility to UC in Israeli Ashkenazi patients with UC contrasting with *Taq*I polymorphisms that favor susceptibility to CD. It seems that in the absence of the VDR, inflammation in the gut is increased, colonic epithelial cell proliferation is dysregulated, and the host tissue fails to satisfactorily maintain gastrointestinal integrity following chemical insult. These data identify vitamin D as a key regulator of gastrointestinal homeostasis and an important player in regulation of the innate immune response.

IFN-γ

IFN-γ seems to be specifically important in CD pathogenesis as suggested by case control studies that showed elevated levels of IFN-γ production in the mucosa in patients with CD, but not in UC[37]. Data indicate that patients with relapsing perianal fistulizing disease have an increased production of IFN-γ measured by *in vitro* cell cultures[38]. Cytokine genotyping study showed that IFN-γ (+874T/A) polymorphism is found in an increased proportion of patients with fistulizing CD[39] probably related to the reduction of tissue repair and migratory potential in fibroblasts apparently influenced by IFN-γ in CD patients[40].

IBD3

Major histocompatibility complex (MHC) genes are located in this region, specifically on the short arm of the human chromosome 6.

With a candidate gene approach the MHC is the most extensively studied region. Two meta-analyses have been carried out to scan for IBD regions that are common for all populations[5,41] in which the highest evidence for linkage to IBD was achieved at the IBD3 locus. There are 3 classes of MHC genes: I, II and III. The antigenic recognizing process in T-lymphocytes from the antigen presenting cells is achieved through the antigenic recognition associated with the MHC gene product[42].

HLA class I

Some not classical genes related to the class I genes such as MHC class I chain-related gene A (MICA) and MHC class I-related chain B (MICB), are expressed in the basolateral cells in the gastric epithelium, fibroblasts, endothelial and dendritic cells. It is known that its expression rises during viral and bacterial infections[43]. Some genetic studies in patients with IBD have found associations with MICA-A6 and HLA-B52 in Japanese patients with UC[44], MICA*010 and HLA-B*1501 in English patients with fistulous CD[23]. MICA and MICB bind to an activating receptor natural killer group 2D (NKG2D) which is expressed on NK cells, T cells and macrophages and the interactions between these receptors may directly stimulate cell cytotoxicity as well as providing costimulation for NK and T cell activation. Several MICA alleles have been shown to alter the binding affinity with NKG2D suggesting they may exert a functional effect on immune activation. In contrast to HLA class II, HLA class I genes show a weak and inconsistent role in IBD. The functional significance of these polymorphisms and the nature of selective forces maintaining them are still being elucidated.

HLA class II

Class II genes are located on the centromeric pole of the short arm of the human chromosome 6 and include HLA-DR, DP and DQ *loci* expressed in a dimeric glycoprotein only in macrophages, activated T-lymphocytes, B-lymphocytes, dendritic, epithelial and endothelial cells, playing a central role in the immune response. Polymorphisms in these molecules are concentrated around specific pockets of the binding groove that interact with critical side-chains or anchor residues of peptides. The different HLA molecules may bind preferentially to different peptides, or bind the same peptide with varying affinities. In IBD the molecular mimicry may exist between the peptides derived from bacterial luminal flora and from self antigens present in the gut, leading to the generation of auto reactive T cells and contributing to disease pathogenesis. The mechanism of cross reactivity is supported by the identification of murine MHC-restricted CD4+ T cells reactive to enteric bacterial antigens that are able to induce colitis by adoptive transfer[45].

In a meta-analysis made by Stokkers *et al*[46], positive associations between UC and HLA-DR2, HLA-DRB1*1502, HLA-DR9 and HLA-DRB1*0103 were found. A study from Mexican population found that HLA-DRB1*0103 allele was associated with UC and its severe manifestations such as colectomy and pancolitis, while HLA-DRB1*15 allele was only associated with pancolitis in Mexican patients with UC.

HLA class III

These genes are located on the 1100 kb section between class I and II genes inside the MHC, and contain about 70

genes. The complement gene block is inherited as a genetic unit known as complotype. Each complotype codifies for the synthesis of complement classic pathway C2, C4A, C4b factors, and alternative pathway B factor, which may suggest that alterations within the region might affect the host's defense system and introduce a complement deficiency. This raises attention when TNFα is thought to play an important role in the pathogenesis of IBD, acting as a potent proinflammatory cytokine with elevated serum and tissue levels in patients with IBD[47-49], and evidence show that there are specific genetic polymorphisms involving TNFα that influence the amount of cytokine produced. Bouma *et al*[50] and Louis *et al*[51] studied the allelic frequency of TNFα gene polymorphisms at -308 position finding that polymorphism in allele 2 was decreased in UC patients as compared to normal controls. It was also found that patients with pancolitis had increased frequency in the TNF-C haplotype[52]. In a Mexican population with UC, the presence of TNF*2 allele was associated with the presence of this disease as compared with healthy subjects (23.7% *vs* 3%, $P = 0.00002$; OR = 10.1; 95% CI = 2.69-26.8)[53]. In Mexican patients with UC, an association was found between complotype SC30 (Bf*S-C2*C-C4A*3-C4B*0) and UC[54], which might suggest that activation of complement system could interfere with the disease pathogenesis.

IBD4

This locus is located on chromosome 14. Evidence for linkage to the adjacent D14S261 and D14S283 *loci* on chromosome 14q11-12 satisfied criteria for confirmed linkage and this region was designated IBD4 locus[55]. Vermeire *et al*[56] in a genome wide scan in a 149 Belgian IBD affected families cohort, demonstrated the existence of IBD4 on 14q11 as a susceptibility *loci*. The IL-25 gene is located within this susceptibility region at 14q11.2. The IL-25 gene is located within this susceptibility region at 14q11.2.

IL-25

Interleukin-25 (IL-25) is a newly identified proinflammatory cytokine that has been shown to promote Th2 responses by inducing cytokines such as IL-4, IL-5 and IL-13, implicated in the initiation of type 2 cytokine-dependent immunity to gastrointestinal infection and limiting proinflammatory cytokine production and chronic intestinal inflammation. IL-25-deficient knockout mice failed to develop a type 2 immune response or eradicate infection. Moreover, chronically infected IL-25 (-/-) mice developed severe infection-induced intestinal inflammation identifying a role for IL-25 in limiting pathologic inflammation at mucosal sites in the gastrointestinal tract[57]. Still more evidence is needed to conclude a precise role of this gene in the IBD susceptibility.

IBD5

The IBD5 locus on chromosome 5q31-33[58] contains the cytokine cluster and is a candidate region for IBD. The IBD5 risk haplotype has been associated with CD, although there have been some suggestions of a weak association with UC as well. Phenotypically this locus has been associated with earlier onset of disease as well as perianal disease[59-62].

Carnitine/Organic cation transporter genes

The organic cation transporter genes OCTN1 and OCTN2 are within a single haplotype block (block 7) of the IBD5 locus and some mutations have been reported within these: L503F (rs1050152) and G-207C (rs2631367) in the *SLC22A4 (OCTN1)* and *SLC22A5 (OCTN2)* genes, respectively, which are associated with the development of CD and also an association with susceptibility to UC has been reported[63]. The presence or combination of these mutations constitutes the TC haplotype, which is associated with ileal, colonic and perianal disease[64,65]. Associations between TC haplotype variants and CD affected sites have been shown in genotype-phenotype studies[66,67]. According to some studies, 1672C→T missense substitution in SLC22A4 and the -207G→C transversion in the SLC22A5 promoter contribute to disease susceptibility by impairing OCTN activity or expression respectively[68]. The risk associated with the OCTN-TC haplotype seems to be only observed in homozygotes and not in heterozygotes[60,63,69], so the carriage of the homozygous OCTN-TC haplotype is likely to be associated with a higher relative risk for colonic disease. The association of the OCTN polymorphisms with CD phenotypes shows a higher frequency of the OCTN-TC haplotype in patients with colonic involvement compared with exclusive ileal disease[60]. It has been reported a moderate increase in the frequency of the TC haplotype among patients without fistulas or stenosis and this is compatible with the negative association with ileal involvement, showing a tendency towards a lower-frequency of ileocecal resection in the presence of at least one OCTN-TC haplotype and might explain the absence of colonic involvement[64]. The impaired eradication of luminal pathogens results in a persistent infection which may constitute a possible mechanism causing IBD.

Drosophila long disc homologue 5 gene

Drosophila long disc homologue 5 gene (DLG5) on chromosome 10q22-23 is a member of the membrane associated guanylate kinase gene family which encodes cell scaffolding proteins and seems to play a role in the maintenance of intestinal epithelial cells, and its mutations have been involved in a rise in intestinal permeability[70]. DLG5 is a widely expressed protein found in many tissues such as the placenta, small bowel, colon, heart, skeletal muscle, liver and pancreas and it is important in signal transduction and epithelial cell integrity. Four haplotypes have been identified associated with IBD in a European cohort[71]. Haplotype A is characterized by the presence of an insertion of thirteen pairs in exon 26. It has been shown to be protective in some case control studies[72], however it is substantially undertransmitted in people with IBD[62]. The haplotype characterized by the haplotype-tagging SNP G113A called Haplotype D, was found substantially overtransmitted in patients with IBD controversially contradictory with another[73] Belgian study where the D haplotype involving the 113A variant was shown to be undertransmitted in flamish patients with

IBD. These apparently contradictory results might yet be compatible with the possibility that DLG5 has a small effect in IBD with heterogeneity in its effect.

ATP-binding cassette or multidrug resistant gene

The multidrug-resistance (MDR1) gene is located on the long arm of chromosome 7 and consists of 29 exons. The total length is 209 kb and 6326 bp. Its product, the P-glycoprotein (Pgp), a member of the ATP binding cassette family, is an integral membrane protein which functions as an energy-dependent efflux pump and reduces the intracellular concentrations of toxins and xenobiotics[74]. Studies show evidence for natural single nucleotide polymorphisms (SNPs) of MDR1 gene and their effects on drug efficiency, toxicity, distribution, absorption and elimination. Two main polymorphisms or variants of this gene have been described, C3435T and G2677T which are associated with IBD in some populations[75,76] and have also been related with the expression of glycoprotein P-170. Variant C3435T was related with the presence of pancolitis in patients with UC in Scotland[77]. However, the frequency of SNPs is low and is different among populations, with the exception of three SNPs in exon 12 (C1236T), exon 20 (G2677T/A) and exon 26 (C3435T), and some of them are correlated with different diseases and clinical characteristics[78]. Glucocorticoid is a potent inhibitor of the T cell activation and a highly effective treatment for IBD[79]. Over-expression produces three molecular mechanisms of glucocorticoid resistance: increase of P-gp and decrease of cytoplasmatic glucocorticoid, dysfunction at the level of glucocorticoid receptor and activation of NFκB, resulting in inhibition of glucocorticoid receptor transcriptional activity. Cucchiara *et al*[80] investigated the predisposition and response to medical therapy of TNFα and MDR1 genes in 200 pediatric patients with CD and 186 UC patients and 347 adults as a control. The 308A allele of the TNF-α gene was increased in both patients with CD and UC, strongly suggesting this polymorphism carries a significant reduction in response to steroid therapy.

IL-6 (-174G/C) polymorphism

IL-6 is a well-studied IBD candidate gene and its polymorphism has been associated with the site of disease in CD. IL-6 levels are higher in patients with active CD as compared to patients with active UC and normal controls[81,82]. A study from Cantor *et al*[39] demonstrated a relationship between IL-6 genotype and the site of CD, showing that patients with the high producer of IL-6 genotype were more likely to have colonic CD. In CD patients IL-6 concentrations also correlate with the disease activity, response to treatment and rate of relapse.

IBD6

In a Canadian linkage scan, a linkage peak of genome-wide scan on chromosome 19p was identified and appeared to confer susceptibility to both CD and UC[61]. Two independent genome-wide linkage studies also determined evidence for linkage to this region and two other meta-analyses of all published genome-wide scans[5,41,83] identified evidence that supports the existence of a locus conferring susceptibility to IBD in chromosomal region 19p, currently known as the IBD6 locus. In order to identify IBD susceptibility alleles in the 19p region two candidate genes DDXL and intracellular adhesion molecule 1 (ICAM-1) were examined in a case-control study with CD and UC patients but no association with either UC or CD was found in 3 single nucleotide polymorphisms in DDXL gene, however a significant association was found between ICAM-1 K469 homozygosity and CD as well as E469 and fistulating disease[84].

IBD7

Located on the short arm of chromosome 1, IBD7 is thought susceptibility genes are residing in this locus. One of these codifies for the transforming growth factor beta 2 (TGF-beta 2) which is a cytokine present in human and bovine milk and plays a critical role in the development of tolerance, prevention of autoimmunity, and in anti-inflammatory responses and is also a potent inhibitor of intestinal epithelial cell (IEC) growth and stimulates IEC differentiation[85-87]. McKaig and colleagues[88] studied the expression of TGF-beta isoforms in isolated and cultured primary human intestinal myofibroblasts from normal controls as well as from UC and CD patients, and determined the responsiveness of these cells to TGF-beta isoforms. Proliferation of myofibroblasts in CD patients was significantly greater than that of myofibroblasts derived from normal and ulcerative colitis tissue, suggesting that it may be related to the development of intestinal strictures, seen frequently as a major feature in CD. The anti-inflammatory attributes of TGF-β3 may be evidenced in a study of children with active intestinal Crohn's disease, who were treated with an oral polymeric diet rich in TGF-β2 as the sole source of nutrition for eight weeks and it was associated with mucosal healing and a down-regulation of mucosal pro-inflammatory cytokines mRNA in both the terminal ileum and colon[89]. However, further investigation on this locus is needed to determine the level of significance related to the pathogenesis of IBD.

IBD8

This gene is located on the short arm of human chromosome 16. There has been evidence of a second chromosome 16 locus (IBD8) independent of NOD2 that overlaps IBD1 on the pericentromeric short arm[90], but yet no studies have been performed for the identification on this locus.

IBD9

The CC-chemokine receptor 5 *(CCR5)* gene located on chromosome 3p21 coincides with this IBD susceptibility locus identified by genome-wide scanning[91]. The CCR5 is the receptor for regulated and normal T-cell expressed and secreted (RANTES), a natural pro-inflammatory cytokine. A 32-bp deletion (A32) in the CCR5 gene results in a nonfunctional receptor found with a high

frequency in Caucasians. They found an association between CCR5delta32 homozygosity and the presence of anal lesions in CD patients with statistical significance[92]. Several genes located in these regions are still under research (Table 1).

GENES INVOLVED IN THE INNATE IMMUNE RESPONSE

Toll like receptors

Rising evidence suggests an essential role of the enteric bacterial flora in the pathogenesis of IBD. Rather than a passive barrier, the intestinal epithelium is an active participant in the mucosal immune response through its expression of proinflammatory genes, secretion of inflammatory cytokines, and recruitment of inflammatory cells in response to pathogenic bacteria and their products[93]. IBD has been increasingly thought to result from an aberrant interaction between the environment and the genetically susceptible host. Specifically, several lines of evidence point to a deregulation of the immune response to a commensal or uncharacterized pathogenic bacterium in the gut[94]. Animal models have demonstrated that genes involved in the regulation of the immune response are likely to play a crucial role in the genetic predisposition to IBD[95]. The family of Toll-like receptors *(TLR)* recognizes pathogen-associated molecular patterns and activates signal transduction pathways of the innate immune response genes including NFκB[95]. The SNPs involved in bacterial recognition are becoming essential in understanding individual responses to bacterial components and defining genetic backgrounds at risk of IBD.

Toll-like receptor 4: The toll-like receptor 4 (TLR4) gene is located on the long arm of human chromosome 9 and it identifies lipopolysaccharides (LPS) on gram-negative bacteria. It has been found strongly upregulated in IBD, and it binds to LPS together with CD14 and by internalization prevents inappropriate NFκB activation[96]. The TLR4 Asp299Gly polymorphism has been associated with CD and UC in a Belgian study[97]. On the other hand this SNP was exclusively related to CD in other series[97-102] and TLR4 polymorphism Thr399Ile was exclusively associated with UC in others[99]. A lipid A-mimetic CRX-526 with antagonistic activity for TLR4, is known to block the interaction of LPS with the immune system, therefore, CRX-526 can prevent the expression of proinflammatory genes stimulated by LPS *in vitro*. This disturbed activation of the innate immune system by bacterial antigens may be crucial in some patients with IBD.

TLR1, TLR2, and TLR6: Pierik *et al*[100] studied the nonsynonymous polymorphisms in other TLR genes in IBD. They found no SNP was involved in disease susceptibility, and a number of variants influenced the disease phenotype, however, they found a positive association between TLR1 R80T and TLR2 R753G and pancolitis in UC. TLR2 and its cofactors TLR1 and TLR6 are involved in the initial immune response to bacteria and recognition of peptidoglycan. This TLR2 is required for recognition of Gram-positive and mycobacterial pathogen-associated molecular patterns (PAMPs) including bacterial lipopeptide lipoteichoic acid (LTA), peptidoglycan (PGN) and the mutations associated are involved in severe mycobacterial infections[103-109]. Further studies have shown that combinations of TLR molecules are required for recognition of certain PAMPs and that specifically, combined expression of TLR2 and TLR6 is required for recognition of PGN[110-114].

It is suggested that TLR1 may be regulated diversely in inflammation to down-regulate or enhance the response to certain TLR2 ligands and that a relative absence of TLR2 protein expression may be important in preventing chronic proinflammatory cytokine secretion in response to commensal Gram-positive bacteria in the gut[95].

TLR5: *TLR5* gene is located on the short arm of human chromosome 1 and is responsible for recognizing a protein named flagellin which is found in intestinal bacteria[113]. Lodes *et al*[113] observed through serological studies a strong response to flagellin in multiple animal models of colitis and synergism has been identified between NOD2 and TLR5 signaling[114]. The dominant negative variant of TLR5 (TLR5-stop) seems to protect against the development of CD and results in significant reduction of IgA and IgE circulating antibodies against flagellin[115], suggesting that pharmacological blockade of TLR5 has potential in the treatment of CD.

NOD1/CARD4 gene

Located on chromosome 7p14, *NOD1/CARD4* gene is one of the three human NOD-LLR proteins that has similar structure to NOD2/CARD15, having only one CARD domain, a central NOD domain and a leucine rich repeat region (LLR). Its function is the recognition of gram negative bacterial products such as γ-glutamine diaminopimelic acid and plays a role in colonic epithelial defense against the intracellular pathogens *E.coli* and *Shigella flexeneri*. Its effector domain is associated with Ripk2 (a CARD-containing interleukin-1 beta converting enzyme-associated kinase) mediating NFκB activation. In a recent study of 556 patients with IBD (294 CD and 252 UC), an association between the variant rs695857 in nucleotides 30, 258 and 950 of NOD1 and the development of IBD was found. Another variant known as rs2907748 in nucleotides 30, 246 and 263 was also associated with the presence of UC and CD and even with the early onset of the disease (< 25 years)[116]. These genetic variants of NOD1 have shown to be associated with disease susceptibility supporting that impaired local immunity might influence bacterial proliferation and aberrant immune responses in the host.

CCL20

CC-chemokine ligand 20 (CCL20) gene is located on the short arm of human chromosome 2 and codifies for the CCL20 cytokine ligand, which is responsible for the chemoattraction of immature dendritic cells that express CCR6 receptor on the intestinal epithelium and on Peyer's plaques[117], and also attracts memory T lymphocytes.

Microarray analysis and PCR-RT quantification have shown a rise in the expression of mRNA from IBD biopsies with inflammation compared to normal biopsies[118]. A study made in Korean UC patients showed that the expression of CCL20 was significantly up-regulated in the peripheral blood mononuclear cells compared with those of normal healthy controls. Interestingly, untreated UC groups expressed higher levels of CCL20 mRNA than treated UC and normal control groups, therefore suggesting that CCL20 could be modulated by anti-inflammatory drugs[119].

Interleukin 11

IL-11 mediates anti-inflammatory effects and is able to downregulate LPS-induced NFκB activation. The IL-11 gene is therefore a good candidate involved in genetic predisposition to IBD. Klein *et al*[120] evaluated the role of IL-11 in IBD, finding decreased expression and a failure to downregulate NFκB expression that could play an important role in the pathogenesis of UC.

Interleukin 18

IL-18 is a pleiotropic cytokine that induces the production of IFNγ and also regulates Th2 cytokines. It seems to be an important cytokine involved in the pathogenesis of IBD, apparently because SNPs at the 5'-end of IL-18 gene might be closely related to the etiology of IBD. Takagawa *et al*[121] found that IL-18 gene promoter polymorphisms may be related to the extent of disease in UC patients.

Interleukin 23

IL-23 is a heterodimeric cytokine composed of a p19 subunit and the p40 subunit of IL-12. It is produced by macrophages and dendritic cells, and activates memory T cells. Interleukin-12 (IL-12) is composed of p35 and p40 subunits and acts as an important factor for the differentiation of naive T cells into T-helper type 1 CD4+ lymphocytes secreting interferon-gamma. Therefore it has been reported that IL-12 is crucial for T-cell-dependent immune and inflammatory responses through the use of IL-12 p40 gene-targeted mice and neutralizing antibodies against p40[122-127]. Apparently IL-12 is a key factor driving Th1 responses and IFN production in the initial phases of an immune response, but conversely IL-12 may play a subsequent immunoregulatory role in late-stage inflammation at a point when IL-23 strongly supports the inflammatory process. IL-23 induces the production of IL-17 by a unique subset of memory T cells. IL-17 is known to stimulate fibroblasts, endothelial cells, macrophages and epithelial cells to secrete multiple pro-inflammatory mediators[128] and the local production of IL-17 causes site-specific activation of inflammatory cells[129-132]. Dendritic cells found in the lamina propria of the small intestine were described as constitutively expressing IL-23[131], whereas IL-23 regulates a highly potent T cell-derived cytokine that has major actions on the immune system. IL-23 specifically stimulates memory CD4+T cells contrasting the IL-12 which is a stimulant for naive CD4+ T cells[129,130]. Studies with IL-23 deficient mice show that IL-23 is essential for the manifestation of intestinal inflammation and a dominant role for IL-23 over IL-12 in central nervous system and joint autoimmune inflammation has been described. These findings point to IL-23, but not IL-12, as the necessary mediator for organ specific autoimmune diseases development. Furthermore, the absence of IL-12 results in more severe disease, reflected in elevated and prolonged expression of proinflammatory cytokines. Yen and colleagues[132] reported that the activation of tissue-homing memory T cells by IL-23 is responsible for chronic inflammatory disease.

CONCLUSION

Genetic research in IBD has provided knowledge about the complexity and heterogeneity of the disease and started to correlate the interactions between genetic and environmental risk factors in IBD; however, the complex genetic background that allows the development of IBD is not fully understood.

Understanding the pathways in which genetic factors influence IBD will uncover pathogenesis of the disease, offer more accurate diagnosis and ultimately lead to the breakthrough of better new drugs and therapies. Most of the important advances toward understanding this process have been identification of specific genetic associations with IBD, which will shed new light on future research of IBD.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Role of bacteria in the etiopathogenesis of inflammatory bowel disease

Nicolas Barnich, Arlette Darfeuille-Michaud

Nicolas Barnich, Arlette Darfeuille-Michaud, Pathogénie Bactérienne Intestinale, Univ Clermont 1, USC INRA 2018, Clermont-Ferrand F-63001 and Institut Universitaire de Technologie en Génie Biologique, Aubière F-63172, France
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Correspondence to: Nicolas Barnich, PhD, Pathogénie Bactérienne Intestinale, USC INRA 2018, Université d'Auvergne, Centre Biomédical de Recherche et Valorisation, 28 Place Henri Dunant, Clermont-Ferrand 63000,
France. nicolas.barnich@u-clermont1.fr
Telephone: +33-4-73177997 Fax: +33-4-73178371
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Abstract

Increased numbers of mucosa-associated *Escherichia coli* are observed in both of the major inflammatory bowel diseases, Crohn's disease (CD) and ulcerative colitis (UC). A potential pathophysiological link between the presence of pathogenic invasive bacteria and genetic host susceptibility of patients with ileal CD is suspected. In CD patients, with increased ileal expression of the CEACAM6 molecule acting as a receptor recognized by type 1 pilus bacterial adhesin, and with the identification of mutations in the NOD2-encoding gene, the presence of pathogenic invasive bacteria could be the link between abnormal ileal bacterial colonization and innate immune responses to invasive bacteria. In a susceptible host, the sequential etiological steps of the disease induced by adherent-invasive *E. coli* (AIEC) are: (1) abnormal colonization *via* binding to the CEACAM6 receptor, which is overexpressed in the ileal mucosa of CD patients; (2) ability to adhere to and to invade intestinal epithelial cells, which allows bacteria to cross the mucosal barrier; (3) survival and replication within infected macrophages in the lamina propria; and (4) induction of tumor necrosis factor-α secretion and granuloma formation.

Key words: Adherent-invasive *Escherichia coli*; Crohn's disease; Inflammatory bowel disease; Ulcerative colitis

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INTRODUCTION

Idiopathic inflammatory bowel diseases (IBDs), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic disorders of the gastrointestinal tract that have a combined prevalence of approximate 150-200 cases per 100 000 population in Western countries[1]. Several lines of evidence suggest that bacteria play a role in the onset and perpetuation of IBD[2-6]. Intestinal bacteria are essential for the development of intestinal inflammation, and are required for the onset of inflammation in numerous knockout models of IBD[7-9]. The pathogenesis of CD is complex and consists of three interacting elements: genetic susceptibility factors such as NOD2/CARD15 and ileal CEACAM6 expression; priming by enteric microflora; and immune-mediated tissue injury[4,6,10-13]. The role of luminal bacteria in the pathogenesis of CD is strongly supported by observations showing that clinical symptoms of CD improve when luminal bacterial levels decrease following intestinal washes and antibacterial drug administration[14-16]. In addition, postoperative exposure of the terminal ileum to luminal contents is associated with increased inflammation in CD, and diversion of the fecal stream is associated with improvement[17].

Studies of luminal bacterial composition in patients with IBD, using culture and molecular biology techniques, have shown a decrease in the number of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* spp. and an increase in pathogenic bacteria such as Bacteroides and *Escherichia coli* (*E. coli*)[18-20]. Such dysbiosis induces a breakdown in the balance between putative species of protective *vs* harmful intestinal bacteria, and may promote inflammation[21,22]. Patients with IBD have higher numbers of mucosa-associated bacteria than control patients[18], and the generalized or localized dysbiosis observed is due to the presence of low numbers of normal bacteria, high numbers of unusual bacteria, and sometimes, a reduction in biodiversity. CD has features that might be the result of a microbial process in the gut. These include onset of infection in Peyer's patches and lymphoid aggregates, and the presence of ulceration, micro-abscesses, fissures, fistulas, granulomas and lymphangitis. Interestingly, the earliest lesions are aphthous ulcers in the intestine, which also occur in some viral and bacterial infections.

Although a number of organisms have been implicated in CD, only two agents, *Mycobacterium paratuberculosis* and *E. coli*, are presently being actively investigated. The theory that *M. paratuberculosis* has a role in CD has some attractive features[23]. Indeed, there are clinical similarities between Johne's disease, a spontaneous *M. paratuberculosis* infection in ruminants, and CD. *M. paratuberculosis* is detected at a greater frequency in CD than in control patients (UC patients and healthy subjects), by culture and polymerase chain reaction (PCR). This organism has been detected in blood and breast milk of patients with CD[24]. The high levels of *E. coli* colonizing the intestinal mucosa in CD patients strongly suggest that it plays a role in the etiopathogenesis of CD.

E. COLI ABNORMALLY COLONIZES ILEAL MUCOSA OF GENETICALLY PREDISPOSED IBD PATIENTS

Bacterial adhesion to intestinal epithelial cells is the first step in the pathogenicity of many organisms involved in infectious diseases of the gut. Adhesion enables the bacteria to colonize the mucosa and to resist mechanical removal from the intestine. Studies on the adherence properties of *E. coli* in CD have yielded the general conclusion that *E. coli* strains are able to adhere to various human cells or cell lines. Fifty-three to 62% of *E. coli* strains isolated from feces of CD patients were able to adhere to buccal cells, compared to only 5%-6% of those isolated from control subjects[25,26]. The comparison of the adhesive properties of *E. coli* strains isolated from the ileum of CD patients and controls has revealed that 80% of *E. coli* strains associated with the ileal mucosa of CD patients preferentially adhered to differentiated Caco-2 cells, which mimic a mature intestinal cell model[20]. This is consistent with the finding that in patients with CD, crypt epithelial cells, which correspond to immature cells, are rarely involved in early lesions[27]. In addition, a correlation between bacterial adhesion to intestinal cells and intestinal colonization has been observed[20]. The presence of high levels of bacteria creates a biofilm on the surface of the gut mucosa in patients with CD and UC[18]. When bacteriologic samples were taken during surgery for CD, *E. coli* was isolated more frequently from the intestinal serosa and mesenteric nodes of CD patients (27% and 33%, respectively) than from those of control subjects[28,29]. Increased numbers of mucosa-associated *E. coli* are observed in CD and UC[18-20,30-33]. Rectal mucosa-associated *E. coli* counts were also higher in active than in inactive UC and CD and controls, and clusters of *E. coli* were observed in the lamina propria in UC and CD specimens, but not in controls[34]. In a study to assess the predominance of *E. coli* strains associated with the ileal mucosa of CD patients, *E. coli* was recovered from 65% of chronic lesions (resected ileum) and from 100% of the biopsies of early lesions (postoperative endoscopic recurrence)[20]. *E. coli* was abnormally predominant (between 50 and 100% of the total number of aerobes and anaerobes) in early and chronic ileal lesions of CD patients[20]. Moreover, in any given patient, healthy and ulcerated mucosa are colonized by *E. coli* strains having the same ribotype profile, which is indicative of uniform colonization, regardless of the inflammatory state of the mucosa[35].

Abnormal colonization of the ileal mucosa is due to increased expression of CEACAM6, a receptor for adherent–invasive *E. coli* (AIEC)[13]. These bacteria have been isolated from ileal lesions of CD patients, and express the type 1 pilus variant, as opposed to the type 1 pilus expressed by *E. coli* MG1655[36]. CD-associated AIEC strains adhere to the brush border of primary ileal enterocytes isolated from CD patients, but not from control patients without IBD. AIEC adhesion is dependent on type 1 pilus variant expression on the bacterial surface[36] and on abnormal CEACAM6 expression on ileal epithelial cells in CD patients[13]. The significantly increased ileal CEACAM6 expression in the uninvolved ileal mucosa of CD patients compared to that in controls without IBD, suggests that patients expressing a basal level of CEACAM6 are genetically predisposed to express that molecule. Additionally, CEACAM6 expression in cultured intestinal epithelial cells is increased after interferon (IFN)-γ or tumor necrosis factor (TNF)-α stimulation, and after infection with AIEC bacteria, which indicates that AIEC can promote its own colonization in CD patients[13]. Accordingly, in patients expressing a basal level of CEACAM6, the presence of AIEC bacteria and the secretion of IFN-γ and TNF-α lead to amplification of colonization and inflammation.

INVASIVES PROPERTIES OF *E. COLI* STRAINS ASSOCIATED WITH CD

Analysis of *E. coli* strains isolated from early or chronic ileal lesions of patients with CD has revealed the presence of true invasive pathogens, since CD-associated bacteria efficiently invade a wide range of human epithelial cell lines, including Hep-2 cells and the intestinal cell lines Intestine-407, Caco-2 and HCT-8[37]. Their uptake is dependent on functioning host-cell actin microfilaments and microtubules[37]. Electron microscopy of epithelial cells infected with CD-associated bacteria has revealed a macropinocytosis-like process of entry, characterized by elongation of the membrane extensions, which surround bacteria at the sites of contact between entering bacteria and epithelial cells. Inside the host cells, CD-associated bacteria survive and replicate in the cytoplasm after lysis of the endocytic vacuole. The invasive process of CD-associated bacteria is unique since it does not possess any of the known genetic invasive determinants described for enteroinvasive, enteropathogenic, and enterotoxigenic *E. coli*, and *Shigella* strains. The major virulence factors of CD-associated AIEC that play a role in their invasive ability are type 1 pili that induce membrane extensions[36], flagella that confer bacterial mobility and down-regulate the expression of type 1 pili[38], outer membrane vesicles that deliver bacterial effector molecules to host cells[39], and outer membrane protein C (OmpC), which regulates the expression of several virulence factors *via* the sigma(E) regulatory pathway[40]. Interestingly, among these virulence

factors, the outer membrane vesicles of *H pylori* and *Pseudomonas aeruginosa* have been reported to induce pro-inflammatory responses[41,42], and bacterial flagellin can interact with Toll-like receptor (TLR) 5 to activate an innate immune response.

The invasive ability of AIEC strains can allow bacteria to translocate across the human intestinal barrier and move into the deep tissues. Consequently, AIEC can interact with resident macrophages and continuously activate immune cells. In addition, patients with CD are more likely to be sensitive to AIEC infection. Indeed, the *NOD2* gene, located on chromosome 16q12, has been identified as the first susceptibility gene for CD[11,12]. NOD2-deficient mice show loss of protective immunity in response to bacterial muramyl dipeptide, and mice are susceptible to *Listeria* infection *via* the oral route[43]. The 3020insC mutant of NOD2 associated with CD has impaired function as a defensive factor against intracellular bacteria in intestinal epithelial cells[44]. Thus, patients carrying NOD2 mutations are unable to control bacterial infections. The mutated NOD2 receptor does not contribute to pro-inflammatory gene transcription in response to bacteria, which results in an inadequate innate response to bacterial invasion and enables bacteria to accumulate. Such a poor innate response can lead to the formation of granulomas and thus, to the activation and perpetuation of a deregulated secondary adaptive response.

AIEC SURVIVAL AND REPLICATION WITHIN MACROPHAGES AND GRANULOMA FORMATION

The search for infectious agents likely to cause CD has focused mainly on intracellular pathogens that have evolved to resist phagocytosis and to persist within macrophages, and which may be involved in chronic antigenic stimulation leading to T-cell and macrophage activation. AIEC strains isolated from CD patients are able to survive and replicate extensively within murine macrophages[45]. At 48 h post-infection, the number of intracellular AIEC bacteria can increase up to 74-fold compared to the initial infection. In contrast to its behavior within intestinal epithelial cells[37], CD-associated bacterial replication does not require bacterial escape into the cytoplasmic compartment[45]. Within J774-A1 macrophages, AIEC bacteria induce the formation of a single spacious vacuole by fusion of initial phagosomes. The behavior of the AIEC strains within macrophages is different from that of other invasive bacteria. In contrast to most invasive bacteria that induce death of infected macrophages[46], no necrosis or apoptosis of AIEC-infected J774-A1 macrophages is observed even after 24 h post-infection[45]. Moreover, in contrast to many pathogens that escape from the normal endocytic pathway, AIEC bacteria are taken up by macrophages within phagosomes, which mature without diverting from the classical endocytic pathway, and share features with phagolysosomes[47]. To survive and replicate in the harsh environment encountered inside these compartments, including acid pH and proteolytic activity of cathepsin D, AIEC have elaborate adaptation mechanisms, for which acidity constitutes a crucial signal, to activate the expression of virulence genes[48]. The major virulence factors of CD-associated AIEC that have a role in their ability to survive and replicate within macrophages are the *htrA* gene that encodes the stress protein HtrA, essential for intracellular replication within macrophages[48], and the *dsbA* gene that encodes the periplasmic oxidoreductase DsbA, essential for AIEC LF82 to survive within macrophages, irrespective of the loss of flagellum and type 1 pilus expression[49]. LF82-infected macrophages release large amounts of TNF-α[45]. This result is in accordance with the fact that several studies have shown that T helper (Th)1 cytokines, such as IFN-γ, TNF-α, and interleukin (IL)-12, are secreted in excess in CD whereas in UC, an atypical Th2 immune response with secretion of IL-4 or transforming growth factor (TGF)-β was observed[50]. Continuous macrophage activation and TNF-α release in CD patients may be due to the sustained multiplication of intracellular AIEC bacteria within phagosomes, and may be involved in the formation of granulomas. Granulomatous inflammation is a histological hallmark of CD and infection with some intracellular bacteria. *E. coli* DNA is present in 80% of microdissected granulomas in CD patients[51]. Granulomatous responses to *E. coli* have been reported in animals, such as granulomatous colitis of boxer dogs or Hjarre's disease in chickens and turkeys. *E. coli* strains were isolated from 100% of granulomas in boxer dogs with colitis[52], and these bacteria resembled CD-associated AIEC in phylogeny and virulence gene profile[53]. In Hjarre's disease, mucoid *E. coli* has been isolated from tuberculoid lesions of the cecum and liver of chickens and turkeys, while intramuscular inoculation of pure bacterial cultures or triturated diseased tissues reproduced the disease[54-56]. Using an *in vitro* model of human granuloma[57], CD-associated AIEC LF82 were reported to induce aggregation of infected macrophages, some of which fused to form multinucleated giant cells and subsequent recruitment of lymphocytes. Analysis of the cell aggregates indicated that they are very similar to the early stages of epithelioid granulomas[58].

PREVALENCE OF AIEC IN IBD

AIEC strains have been found to be highly associated with ileal mucosa in CD patients[56]. Such pathogenic strains were isolated from ileal specimens of 36.4% of CD patients *vs* 6% of controls. In colonic specimens, AIEC strains were found in 3.7% of CD patients, 0% of UC patients, and 1.9% of controls. These strains are preferentially found in early recurrent lesions after surgery, thus indicating their role in the initiation of inflammation, and not just as secondary invaders. Another study has shown that mucosa-associated *E. coli*, which accounted for 53% of isolates, were more common in CD (43%) than in non-inflamed control patients (17%), while intramucosal *E. coli* were found in 29% of CD patients *vs* 9% of controls[30]. These studies support a central role for mucosa-associated AIEC in the pathogenesis of CD[30,56], since the translocation of these pathogenic bacteria through the intestinal mucosa may be a crucial step in the propagation of the inflammatory process.

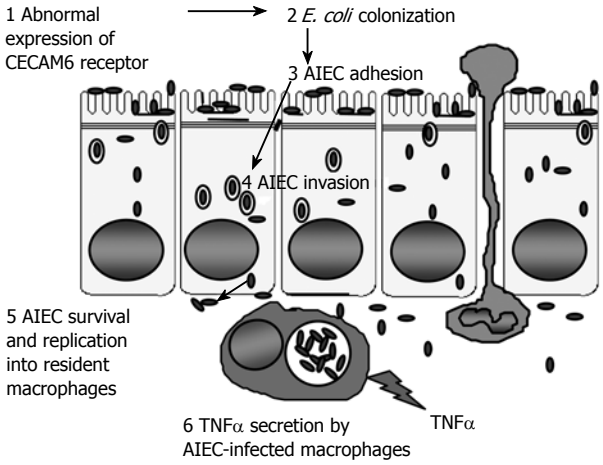

Figure 1 Sequential steps of the mechanisms of disease induced by AIEC bacteria: (1) abnormal expression of CEACAM6 in ileal mucosa of CD, inducing (2) AIEC colonization, (3) adhesion and (4) invasion, which allow the bacteria to cross the mucosal barrier. AIEC bacteria can (5) survive and replicate within infected macrophages in the lamina propria, and (6) induce TNF-α secretion.

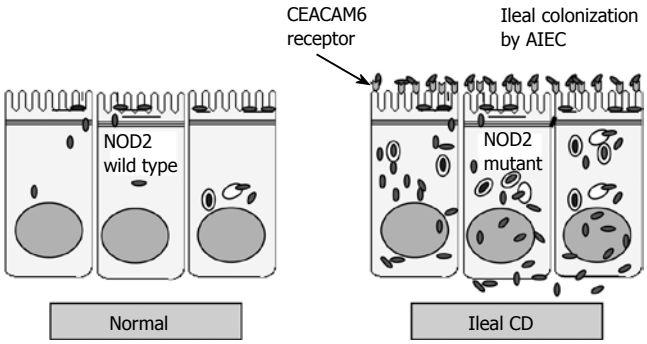

Figure 2 The infection cycle of AIEC may depend upon the ability of these pathogenic bacteria to colonize the gastrointestinal tract of genetically predisposed patients. Patients at high risk for developing severe ileal CD are those who overexpress CEACAM6 in the ileal mucosa, which allows AIEC colonization, and express the NOD2 3020insC mutant which has an impaired function as a defensive factor against intracellular bacteria in intestinal epithelial cells.

CONCLUSION

Various factors lend credence to the theory that AIEC is intimately linked to the etiopathogenesis of ileal CD. The high prevalence of AIEC in patients with ileal CD may be the first step in the establishment of a modified Koch's postulate that takes into account the genetic susceptibility of the host[30,56]. A possible role for AIEC in the etiopathogenesis of CD in susceptible hosts is summarized in Figure 1. The sequential steps involved in the induction of disease by the bacteria are: (1) abnormal colonization *via* binding to the CEACAM6 receptor, which is overexpressed in the ileal mucosa of CD patients[13]; (2) ability to adhere to and to invade intestinal epithelial cells, which allows bacteria to cross the mucosal barrier[37]; (3) survival and replication within infected macrophages in the lamina propria; and (4) induction of TNF-α secretion[45] and granuloma formation[58].

AIEC strains could colonize the ileal mucosa of CD patients by binding to CEACAM6, translocate across the human intestinal barrier to move into deep tissues, and once there, continuously activate immune cells. Patients having a high risk for developing severe ileal CD may be those who, in addition to expressing a variant of the NOD2 intracytoplasmic receptor[11,12], overexpress CEACAM6 at the surface of the ileal mucosa[13] (Figure 2). Host innate immune receptors that can be activated by AIEC components are mainly the transmembrane receptor TLR2 and the intracellular receptor NOD2. NOD2 is a negative regulator of the TLR2-mediated Th1 response, while the NOD2 3020insC mutation associated with CD is unable to inhibit TLR2 signaling, which skews the system toward an overactive Th1-mediated response[59]. This result provides a compelling explanation for why people carrying the NOD2 mutation might develop CD in response to abnormal colonization by AIEC[60]. The treatment of severe ileal CD could evolve from being almost exclusively surgical to management that places much greater emphasis on medical therapy, such as immunomodulators and anti-TNF-α agents, and also on antibiotic or probiotic treatments.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Innate immunity in inflammatory bowel disease

Jesus K Yamamoto-Furusho, Daniel K Podolsky

Jesus K Yamamoto-Furusho, Daniel K Podolsky, Gastrointestinal Unit and Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, United States
Correspondence to: Jesus K Yamamoto-Furusho, MD, PhD, Gastrointestinal Unit, Massachusetts General Hospital, 55 Fruit Street, Boston, Massachusetts 02114, United States. kazuofurusho@hotmail.com
Telephone: +1-617-7267411 Fax: +1-617-7242136
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Abstract

The human intestinal tract is home to an enormous bacterial flora. The host defense against microorganisms can be divided into innate and adaptive immunity. The former is the most immediate line of response to immunologic challenges presented by bacteria, viruses, and fungi. The mucosal immune system has evolved to balance the need to respond to pathogens while co-existing with commensal bacteria and food antigens. In inflammatory bowel disease (IBD), this hyporesponsiveness or tolerance breaks down and inflammation supervenes driven by the intestinal microbial flora. Bacteria contain compounds and are recognized by a variety of receptors, including Toll-like receptors (TLRs) and NODs (a family of intracellular bacterial sensors) and are potent stimuli of innate immune responses. Several mutations in these receptors have been associated with development of IBD.

Key words: Innate; Immunity; Toll-like receptors; Inflammatory bowel disease

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises two major forms of chronic inflammation of the intestine, Crohn's disease (CD) and ulcerative colitis (UC).

IBD is currently presumed to result from the complex effect of diverse genes conferring risk of disease and environmental factors, which when combined lead to an aberrant inflammation response. Recent evidence suggests that innate immune responses play an important role in initiating the inflammatory cascade and subsequent characteristic pathological adaptive immune responses[1]. The innate immune response is the first line of defense for microbial infections. In addition to genetic factors in IBD, numerous studies have implicated a key role of the intestinal microbiota in patients with IBD[2-4]. The contribution of luminal microbes to the pathogenesis of IBD is highlighted by reports that surgical diversion of the fecal stream effectively resolves CD inflammation distal to the surgical site[2].

The human intestinal tract mucosa is exposed to an enormous microbial flora. A single layer of epithelial cells separates the host tissues from luminal bacteria. Innate immune mechanisms are involved in this relationship, and likely contribute significantly to the protection of the host from invasion by luminal bacteria and provide a rapid response to pathogens. Immediate activation of innate immunity relies on the detection by the host of conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs), comprising diverse molecules from bacteria and viruses such as lipopolysaccharide, peptidoglycan, flagellin and lipoproteins[5].

Understanding of innate immunity has accelerated enormously with the discovery of many microbial sensors called "pattern recognition receptors" (PRRs). The toll-like receptor (TLR) and NOD receptor families of PRRs appear to play essential roles in mucosal homeostasis and alterations contribute to the pathogenesis of IBD.

THE TLR FAMILY

The mammalian TLR family consists of 13 mammalian members: each TLR having its intrinsic signaling pathway and inducing specific biological responses against microorganisms. Recognition of microbial components by TLRs triggers activation of signal transduction pathways, which then induce dendritic cell maturation and cytokine production, resulting in development of adaptive immunity[5].

Table 1 summarizes TLRs and the different molecular patterns associated with a broad range of microbes that they recognize. The activation of TLR signaling pathways originates from the cytoplasmic TIR domains. Four TIR domain-containing adaptors (MyD88, TIRAP/MAL, TRIF, and TRAM) play an important role in TLR signaling

Table 1 Molecular pattern recognition of NODs and TLRs

Receptor	Microbial motifs
NLR family	
NOD1	Lanthionine meso-diaminopimelic acid (meso-DAP) γ-D-Glu-meso-diaminopimelic acid (iE-DAP)
NOD2	Muramyldipeptide (MDP)
TLR family	
TLR1	Triacyl lipopeptides
TLR2	Lipoprotein, lipopeptides ($Pam_3CysSerLys_4$)
TLR3	dsRNA
TLR4	LPS
TLR5	Flagellin
TLR6	Diacyl lipopeptides
TLR7/TLR8	ssRNA
TLR9	Non-methylated CpG DNA
TLR11	Component of uropathogenic bacteria

pathway. These adaptors are associated with TLRs through homophilic interaction of TIR domains. Each TLR mediates distinctive responses in association with a different combination of these adapters[6].

THE NLR FAMILY

The mammalian NLR family comprises more than 20 members whose defining molecular characteristic is a C-terminal leucine-rich repeat (LRR) domain, a central nucleotide-binding NACHT domain and an N-terminal protein-protein interaction domain composed of a CARD (caspase activation and recruitment domain). Several studies have shown that several NLRs are necessary sensors of specific PAMPs.

The first NLRs reported to have a direct function as intracellular PRRs were NOD1 (CARD4) and NOD2 (CARD15). NOD2 detects muramyl dipeptide, the largest molecular motif common to Gram-negative and Gram-positive bacteria[7,8]. In contrast, NOD1 senses peptidoglycan containing meso-diaminopimelic acid (meso-DAP), which is more commonly found in Gram-negative bacteria[9,10].

NALP3 is a pyrin domain-containing NLR that activates the caspase-1 leading to interleukin 1β (IL-1β) and IL-18 processing, this protein is involved in sensing microbial components. In addition to NODs and NALP3 proteins, Ipaf (CARD12) and Nalp1 are intracellular sensors that detect intracellular flagellin, leading to inflammasome activation through a TLR5-independent pathway[11].

The signaling pathways downstream of NLRs include the NF-κB pathway, for NOD1 and NOD2, and activation of the caspase 1 inflammasome, for NAPLPs, Ipaf and Naip. NOD1 and NOD2 rapidly form oligomers and then transiently recruit receptor-interacting protein 2 (RIP2) through CARD-CARD interactions. The complex NOD-RIP2 then recruits the inhibitor of NF-κB kinase complex, which leads to activation of NF-κB. Several studies have shown that Ipaf and Naip can participate in the formation of inflammasomes (NALP1, NALP3 and Ipaf) that form in response to the detection of specific molecular motifs. It has been speculated that the formation of large protein complexes in a given inflammasome is sufficient to trigger caspase-1 activation[12].

NLRs are important in macrophage-mediated detection and control of bacterial infection *in vitro*. Ipaf is required for caspase-1 activation and IL-1β secretion in macrophages exposed to Gram-negative pathogen *Salmonella typhimurium*[13]. Cytosolic recognition of *Salmonella typhimurium* and *Legionella pneumophila* flagellins by Ipaf results in the induction of macrophage cell death and IL-1β secretion.

CONNECTION OF NOD AND TLR PATHWAYS

Intersection between TLR and NOD2 pathways is suggested by reports of synergistic induction of proinflammatory cytokines such as TNFα and IL-1β upon costimulation with MDP and specific TLR ligands[14,15]. MDP also substantially upregulated secretion of TNFα and IL-1β induced by ligands to five different TLRs ligands, TLRs 2, 4, 5, 7 and 9: ($Pam_3CysSerLys_4$, LPS, Flagellin, MALP-2 and R-848, respectively). Of note, these effects were observed in the presence of the most common NOD2 mutants associated with CD. In studies using mice lacking NOD2, Watanabe *et al* observed reduced responses to MDP, but enhanced responses to the TLR2 ligand peptidoglycan e.g., increases in IL-12. They interpreted these findings to suggest that the NOD2 signaling pathways normally down-regulate the TLR2 pathways. In their model, loss of function mutation of NOD2 together with TLR2 signals delivered by other bacterial products could result in enhanced cytokine responses to commensal bacteria by macrophages[16]. These findings suggest that interaction between NOD2 and specific TLR pathways may represent an important modulatory mechanism of innate immune responses, which is altered in some patients with CD.

TLRS IN IBD

TLRs are abundantly expressed on the surface of monocytes, macrophages, dendritic and epithelial cells. Alterations of TLR3 and TLR4 expression by intestinal epithelial cells have been described in IBD[17], suggesting that there is differential expression of TLR family members. Thus, primary intestinal epithelial cells of normal, non-diseased mucosa constitutively express TLR3 and TLR5, whereas TLR2 and TLR4 are present in much lower amounts. In active IBD, the expression of TLR3 and TLR4 was differentially modulated in the intestinal epithelium. TLR3 was significantly down-regulated in active CD but not in UC. In contrast, TLR4 was strongly up-regulated in both UC and CD. TLR2 and TLR5 expression remained unchanged in IBD.

Two common polymorphisms of TLR4 (Asp299Gly and Thr399Ile) have been described in humans. Asp299Gly has been associated with reduced responsiveness following lipopolysaccharide stimulation[18]. These polymorphisms have been associated with the development of CD and UC in Caucasian populations[19-21].

Recently, Pierik *et al*[22] showed that TLR1 R80T and TLR2 R753G polymorphisms were associated with pancolitis in UC patients, while a negative association

was observed between TLR6 S249P and proctitis in patients with UC. These results suggest that TLR2 and its co-receptors TLR1 and TLR6 are involved in the initial immune response to bacteria in the pathogenesis of IBD.

An important immune stimulatory effect mediated by the TLR family (TLR9) is induced by non-methylated CpG motifs found in bacterial DNA. In animal models of colitis, administration of CpG was able to ameliorate disease activity[23].

NODS IN IBD

Specific mutations of the NOD2 gene have been definitively associated with increased susceptibility to ileal Crohn's disease in Western (but not Asian) populations: Arg702Trp, Gly908Arg, and leu1007fsinsC (a frameshift mutation that truncates the carboxy terminal 33 amino acids)[24,25]. Heterozygous carriage of the risk alleles confers a 2-4 fold increased risk, and homozygotes or compound heterozygotes have a 20-40 fold increased risk[26]. More than 90% of all CD associated mutations are located in the LRR domain, suggesting that these may affect the function of NOD2 with respect to bacterial recognition and signaling. Transient transfection experiments indicate that CD-associated NOD2 mutants no longer activate NF-κB in response to MDP[27,28], which suggests that defective NF-κB activation facilitates infection of the lamina propria by enteric bacteria.

NOD2 mutants produce selective functional defects in leukocytes of patients with CD as shown by van Heel *et al*[8] who analyzed cytokine expression of peripheral blood mononuclear cells after exposure to MDP. In PBMC from CD patients the NOD2 ligand induced little TNFα and IL-1β, but strong IL-8 secretion. Furthermore, monocytes isolated from CD patients carrying the 1007fs (3020insC) mutation were reported to exhibit defects in the production of the proinflammatory cytokines, TNFα, IL-6 and IL-8, as well as the anti-inflammatory cytokine IL-10[29]. Dendritic cells derived from CD patients homozygous for leu1007fsinsC also fail to up-regulate the costimulatory molecules CD80 and CD86 in response to MDP and lack production of cytokines such as TNF-α, IL-12 and IL-10[30].

Evidence that NOD2 functions as an antibacterial factor in intestinal epithelial cells was demonstrated in Caco-2 cells stably expressing wild type NOD2 when infected with *Salmonella typhimurium*. This protective effect was absent in cells expressing a most common mutant NOD2 associated with CD (3020insC)[31].

NOD2 mutations in CD patients are also associated with diminished mucosal α-defensin expression[32]. Decreased β-defensin 1 and the lack of induction of both inducible antimicrobial peptides β-defensins 2 and 3 in CD could result in enhanced bacterial invasion and perhaps survival[33].

A study of 556 patients with IBD (294 CD and 252 UC) reported an association between the variant (rs695857) in nucleotides 30, 258 and 950 of the *NOD1* gene and the development of IBD. Another variant known as rs2907748 in nucleotides 30, 246 and 263 was also associated with the presence of UC and CD, particularly early onset of the disease (< 25 years)[34].

CROHN´S DISEASE

Marks *et al*[35] have provided provocative evidence that CD patients possess a generalized impaired innate immune response as reflected by diminished response to intradermal injection of killed bacteria as well as trauma of the skin or the intestine. When killed bacteria were injected into the forearms of CD patients, there was less blood flow to the injection site than non-CD patients. They also found that CD patients had reduced neutrophil accumulation and interleukin-8 (IL-8) production at sites of tissue trauma in the intestine and skin, although these findings need corroboration. This study supports the idea that CD may in some way be associated with relative inability to mount an acute inflammatory response compared to normal individuals.

Recent studies have suggested that the CD-associated NOD2 mutants might confer a milder defect in innate immune response than well-described innate immune deficiencies such as chronic granulomatous disease[8]. Rather than suppressing the secondary T-cell response, a different approach aims to normalize innate immune function through qualitative augmentation of neutrophil, macrophage and dendritic cell function. Clinical trials of granulocyte colony stimulating factor (G-CSF, specifically filgrastim) and GM-CSF (sargramostim) have suggested a benefit, although definitive evidence is not yet available[36,37]. However, G-CSF has a more limited effect on the innate immune system, acting primarily on neutrophils. GM-CSF is more widely and potently active, targeting a variety of cell types including not only neutrophils and monocytes as effector cells, but also intestinal epithelial cells that have receptors for GM-CSF.

CONCLUSION

The innate immune system is the first line of defense and provides a rapid response to pathogens. Elicitation of an innate immune response to bacterial products is mediated through families of pattern recognition receptors including the cell surface TLRs and cytosolic NODs that mediate the activation of NF-κB. Some mutations, TLRs, and NODs produce defects in sensing of pathogens and predispose the host to recurrent infections as well as perpetuation of chronic intestinal inflammation.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease

Mayumi Kawada, Atsuko Arihiro, Emiko Mizoguchi

Mayumi Kawada, Atsuko Arihiro, Emiko Mizoguchi, Gastrointestinal Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, United States
Emiko Mizoguchi, Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, United States
Supported by National Institute of Health grants, No. DK64289, DK74454, and DK43351), IBD grants from the Eli and Edythe Broad Medical Foundation
Correspondence to: Emiko Mizoguchi, MD, PhD, Assistant Professor, Harvard Medical School, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114, United States. emizoguchi@partners.org
Telephone: +1-617-7267892 Fax: +1-617-7263673
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Abstract

Inflammatory bowel disease (IBD), the most important being Crohn's disease and ulcerative colitis, results from chronic dysregulation of the mucosal immune system in the gastrointestinal tract. Although the pathogenesis of IBD remains unclear, it is widely accepted that genetic, environmental, and immunological factors are involved. Recent studies suggest that intestinal epithelial defenses are important to prevent inflammation by protecting against microbial pathogens and oxidative stresses. To investigate the etiology of IBD, animal models of experimental colitis have been developed and are frequently used to evaluate new anti-inflammatory treatments for IBD. Several models of experimental colitis that demonstrate various pathophysiological aspects of the human disease have been described. In this manuscript, we review the characteristic features of IBD through a discussion of the various chemically induced experimental models of colitis (e.g., dextran sodium sulfate-, 2,4,6-trinitrobenzene sulfonic acid-, oxazolone-, acetic acid-, and indomethacin-induced models). We also summarize some regulatory and pathogenic factors demonstrated by these models that can, hopefully, be exploited to develop future therapeutic strategies against IBD.

Key words: Inflammatory bowel disease; Experimental colitis; Dextran sodium sulfate; Trinitrobenzene sulfonic acid; Oxazolone; Pathogenesis

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INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), represents a chronic, relapsing and remitting inflammatory condition that affects individuals throughout life[1]. No completely effective therapeutic strategy has been established because the etiology of IBD remains largely unknown, although there has been extensive research on its pathogenesis. However, recent advances in the understanding of the pathophysiology of IBD have provided some clues for developing potentially helpful therapeutic tools.

Within the past two decades, several models of experimental colitis have been reported that demonstrate various pathophysiological aspects of human IBD. While no model serves as a complete surrogate for the human disease, some characteristically pathological features are open for investigation, depending on the method used to induce the experimental colitis. Experimental models of colitis enable us to dissect the pathogenic components during different phases of colitis, including acute, recovery and chronic phases. They also enable us to identify some pivotal immunological processes, as well as novel genes that are intimately involved in disease susceptibility.

In this review, we mainly focus on the role of functionally distinct factors, including immune cells, cytokines/chemokines, receptors/ligands, transcriptional factors, and enzymes/hormones, which maintain the homeostatic balance in the colon during the development of acute and chronic inflammation.

DSS-INDUCED COLITIS

The dextran sodium sulfate (DSS) model, originally reported by Okayasu *et al*[2] has been used to investigate the role of leukocytes in the development of colitis. Oral administration of 5% DSS in drinking water can induce not only acute, but also chronic colitis. One cycle of 3%-5% DSS administration for 5-7 d, followed by

regular water, results in extensive injury with complete crypt depletion (mainly basal crypt) and relatively slow regeneration of colonic epithelium. This regeneration is much slower than in other acute injury models, which use toxic substances such as acetic acid and ethanol[3]. The clinical features of this model include weight loss, loose stools/diarrhea, and rectal bleeding. Histopathological analysis typically reveals extensive crypt and epithelial cell damage, significant infiltration of granulocytes and mononuclear immune cells, and tissue edema, often accompanied with severe ulceration. In fact, because of the massive edema and subsequent ulceration during the acute phase, some researchers have wrongly used the DSS-induced colitis model by interpreting it as a model for human UC; however, this colitis is a simple model of acute chemical injury rather than chronic inflammation. Pathological scoring is generally performed on the distal segment of the colon, which is the most severely affected portion[3]. Histopathology, by hematoxylin and eosin staining, is scored based on three parameters: severity of inflammation (none, mild, moderate, severe), extent of inflammation (none, mucosa, mucosa and submucosa, transmural), and crypt damage (none, basal one-third damaged, basal two-thirds damaged, crypt lost but surface epithelium present, crypt and surface epithelium lost). It is noteworthy that long-term DSS administration produces colorectal carcinoma, which is similar to the dysplasia-carcinoma sequence seen in the course of cancer development in human UC[4].

Acute mucosal damage can be observed in both wild-type and severely combined immunodeficiency (scid) mice, which indicates that acquired immune responses are not involved in the induction of DSS-induced colitis[5]. The lesions observed in scid mice have been associated with increased production of macrophage-derived proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. While the role of luminal bacteria in the pathogenesis of DSS-induced colitis is unclear, this colitis can be ameliorated by treatment with antibiotics that are clinically effective in patients with IBD[1], which suggests the importance of commensal bacteria in the development of colitis[6]. Although the earliest change of acute DSS-induced colitis is a progressive disruption of colonic crypts during the chronic phase (14 d after stopping DSS), macrophages and $CD4^+$ T cells are more prominent in areas of wound healing in the basal portions of the lamina propria (LP). These $CD4^+$ T cells secrete increased levels of interferon (IFN)-γ and IL-4, which suggests that chronic immune activation mediated by both Th1 and Th2 cells play a pathogenic role in chronic DSS-induced colitis[7].

2,4,6-TRINITROBENZE SULFONIC ACID (TNBS)-INDUCED COLITIS

In 1995, Neurath *et al* described a novel murine model of intestinal inflammation induced by intrarectal administration of hapten reagent TNBS in ethanol solution. Simultaneous administration of TNBS and ethanol is required to induce TNBS colitis, because ethanol disrupts the epithelial layer and exposes the underlying LP to bacterial components. Intestinal inflammation induced by intrarectal administration of TNBS has many of the characteristic features of CD in humans, including severe transmural inflammation associated with diarrhea, rectal prolapse, weight loss, and induction of an IL-12-driven inflammation with a massive Th1-mediated response[8]. Interestingly, prior oral administration of TNBS in the form of trinitrophenol-haptenated colonic protein (TNP-CP) prevents colitis induced by intrarectal administration of TNBS[9,10]. The preventive effect is due to the induction in the LP of regulatory cells consisting of $CD4^+$ T cells that produce transforming growth factor (TGF)-β after oral administration of TNP-CP[10].

The susceptibility to TNBS colitis varies between different mouse strains; SJL and BALB/c are susceptible, whereas C57Bl/6 and 10 mice are resistant. The susceptibility has been shown to be related to a genetically determined high IL-12 response to the lipopolysaccharide (LPS) locus on chromosome 11 in SJL/J mice[11]. In a recent study, te Velde and colleagues compared gene expression profiles in the colons of three different models of colitis (DSS, TNBS and $CD45RB^{high}$ T-cell transfer models)[12]. As a result, a restricted number of genes were either up- or down-regulated in the TNBS colitis (21 genes) model compared to DSS-induced colitis (387 genes) and $CD45RB^{high}$ transfer model (582 genes)[12]. Of the 32 genes known to change transcriptional activity in IBD (*TNF, IFN-γ, Lt*β*, IL-6, IL-16, IL-18R1, IL-22, CCR2* and *7, CCL2, 3, 4, 5, 7, 11, 17* and *20, CXCR3, CXCL1, 5* and *10, Mmp3, 7, 9* and *14, Timp1, Reg3*γ*, Pap, S-100a8, S-100a9, Abcb1,* and *Ptgs2*), two (*Mmp14* and *Timp1*) are up-regulated in TNBS, 15 (*IL-6, IL-16, IL-22, CCL2, 3* and *11, CXCL1* and *5, Mmp3* and *14, Timp1, Reg3*γ*, Pap, S-100a9,* and *Ptgs2*) are up- or down-regulated in DSS, and 30 (except for *CCL11 and Timp1*) are up- or down-regulated in the CD45RB transfer colitis models. The study suggests that the pattern of gene expression in these colitis models closely reflects altered gene expression in human IBD[12].

OXAZOLONE COLITIS

In contrast to TNBS, which leads to colitis driven by a Th1-polarized type of T-cell response, administration of another haptenating agent, oxazolone, leads to a colitis associated with a Th2-polarized type of response. This model is induced by the rectal administration of oxazolone suspended in an ethanol vehicle. Although the SJL/J strain of mice was utilized in the original description[13], over half of the later studies have been performed using the C57Bl/6 strain. The induction of colitis in the C57 strain requires a presensitizing treatment, since this strain is resistant to haptenating agents[14]. For presensitization, 4.5 mg - 6 mg of oxazolone in 100% ethanol is injected into the abdominal wall of mice, followed by intrarectal administration of various doses of oxazolone in 50% ethanol after 5 d.

Oxazolone colitis is limited to the distal part of the colon, in contrast to TNBS colitis that is characterized as pan-colitic. Microscopically, the inflammation of oxazolone colitis manifests as relatively superficial ulceration[13]. An IL-4-driven Th2-type of response is predominant and is

Table 1 Pathogenesis of IBD models in DSS colitis

Pathogenic factors	
Categories	Factors (References)
Chemokines/cytokines	Migration inhibitory factor[114], LIX[115], L-18[33], CCR5[116], IL-1[117]
Adhesion molecules	CD98[118], β2 integrins (CD18/11a)[83], Integrin α1β1[81], VCAM-1[119]
Transcriptional factors	STAT3[74]
Toll like receptors and ligands	CpG motifs[92], Flagellin/TLR5[90]
Enzymes	Chitinase 3-like-1[102], Carbonic anhydrase Ⅳ[100], Eosinophil peroxidase[120], Caspase-1[105]
Hormones	Adiponectin[112], Resistin-like molecule β[121], Leptin[113], Osteopontin[122], Activins[123]
Others	Galanin-1 receptor[124]
Regulatory factors	
Categories	Factors (References)
T cells	γδT cells[23,24]
Cytokines/chemokines	BFGF[51], FGF2[125], TGF-α[46], TFF2[53], ITF[54], HGF[47,49]
Transcription factors	SOCS3[74], Nrf2[126], PPARγ[76,77], PPARδ[76]
Adhesion molecules	B2 integrins (CD11β)[83]
Receptors	TLR4[87], PG receptor EP-4[95], Pregnane X Receptor[127]
Enzymes	COX-2[94,96], COX-1[94], Matrix metalloproteinase-2[128]
Hormones	Estrogen[129], Growth hormone[130], Adiponectin[110]
Neuronal factors	Vagus nerve[131], IRE1β[132], Neurotensin[133]
Lipid-associated molecules	Lipoxin A4[134], Apolipoprotein A-Ⅳ[135]
Others	Dietary glycine[136], Follistatin[123], Bacterial superantigens[137], Thioredoxin-1[138]

characterized by increased IL-4/IL-5, but normal IFN-γ production. The inflammation is prevented by the systemic co-administration of intraperitoneal anti-IL-4 antibody. The proinflammatory Th2-dominant cytokine response is regulated by TGF-β, which limits both the extent and duration of the disease. The histological features and inflammatory distribution of oxazolone colitis resemble human UC[13].

OTHER CHEMICALLY-INDUCED COLITIS MODELS

In a search for novel experimental models of acute IBD, MacPherson and colleagues have found that intrarectal administration of 3%-5% acetic acid induces acute colitis in the distal part of the colon in rats[15]. The initial injury consists of epithelial necrosis and edema that variably extends into the LP, submucosa, or external muscle layers. Epithelial injury is mainly caused by organic acids specifically because hydroxyl chloride (pH 2.3) does not generally induce acute colitis[4]. In mice, administration of acetic acid within 4 h results in colonic epithelial destruction without inflammation, which is then followed by an influx of acute inflammatory cells, and reaches its maximum intensity at 12 h. The inflammatory response is caused by non-specific factors after disruption of the epithelial barrier. The chemical injury heals within days in mice or 2-3 wk in rats[16].

Whereas acetic acid produces acute inflammation restricted to the colon, another pro-inflammatory agent, indomethacin, has been used to induce acute ileitis. Fasted rats are treated subcutaneously with indomethacin 7.5 mg/kg in sterile sodium bicarbonate, which leads to an acute inflammatory response characterized by multiple deep, longitudinal ulcers in the distal jejunum and proximal ileum. This acute response reaches its maximum intensity at 24 h and is completely resolved within 7 d, whereas two daily subcutaneous injections of indomethacin produce a chronic inflammation that lasts at least 2 wk[17]. Luminal bacteria and their products significantly contribute to the exacerbation and perpetuation of the chronic phase of indomethacin-induced inflammation.

These models have the advantage of being easy to initiate and therefore would be useful in the initial screening of new drugs for acute epithelial injury. However, the injury in the first 24 h is nonimmunologic and thus is not suitable for drug therapy trials for human IBD.

FACTORS INVOLVED IN THE PATHOGENESIS OF THE MAIN CHEMICALLY INDUCED COLITIS MODELS

In the following section, we focus more on the factors involved in the fine balance between pathogenic and regulatory factors in the pathogenesis of DSS- (Table 1), TNBS- (Table 2), and oxazolone- (Table 3) induced colitis.

T cells

$CD4^+$ T cells play a key role in the development of most T-cell-mediated IBD models. For example, the increased production of IFN-γ, mainly produced by $CD4^+$ T cells, is detected in most models of Th1-mediated colitis[18]. By contrast, IL-4 and IL-13, produced by natural killer (NK) T cells, have been shown to play a key role in the pathogenesis of Th2-mediated colitis, including oxazolone-induced colitis[19]. NK1.1 positive lymphocytes are also essential for alleviation of TNBS-induced colitis in the presence of peripheral tolerance[20].

Although $CD8^+$ T cells represent a major T-cell subset, there is little information available regarding the role of $CD8^+$ T cells in the pathogenesis of colitis. $CD8^+$ T cell receptor (TCR)-positive $V\beta14^+$ T cells, which are increased in the LP and have a cytotoxic effect[21], have a pathogenic role in the development of TNBS-induced colitis.

By contrast, TCRγδ T cells are an evolutionarily

Table 2 Pathogenesis of IBD models in TNBS colitis

Pathogenic factors	
Categories	Factors (References)
T cells	Th1[8], $CD8^+$ TCR $V\beta14^+$ T cell[21], CEACAM1[27]
Cytokines/chemokines	IL-12[8,30], IFN-γ[8,34], IL-18[31,32,139], IL-6[73], IL-16[140], IL-17[38], TNF-α[29,141], MIP-α[142], MIP-3α[143]
Receptors	CD40[57,58], CD44v7[144], FcεRI[145], GITR[63,64], Complement receptor 3[146]
Transcription factors	NF-κB p65[65,67,147], RICK[69,70], MAPK p38[70], Smad7[72], Smad3[148]
Adhesion molecules	Integrinα1β1[80]
Enzymes	Poly (ADP-ribose) synthetase[149,150], Inducible nitric oxide synthase[151], Angiotensinogen[152], Vanin-1[107]
Hormones	Leptin[113], Ghrelin[153], Adiponectin[112]
Others	Geneticfactors[11], Glycolipid[154]
Regulatory factors	
Categories	Factors (References)
T cells	TCRγδ[25,26], NK1.1[20,155,156]
Cytokines/chemokines	TGF-β[10,44,45], IL-10[44,157,158], IL12 p40[34], IL12 p40-IgG2b[159], IL-2-IgG2b[160], IL-23[39], HGF[48], BFGF[51]
Receptors	PAR-2[61], TNFR1[56]
Transcription factors	STAT5b[161], Interferon regulatory factor-1[162], PPARγ[75]
Enzymes	Indoleamine 2, 3-dioxygenase[163]
Hormones	Adrenocortical hormones[164,165], NCX-101[166]
Neurotransmitters	Vasoactive intestinal peptide[167,168], μopioid receptor[169]
Lipid mediators	Lipoxin A4[170], Marine[171]
Bacteria and parasite related factors	Yersinia pseudotuberculosis[172], Lactic acid bacteria[173,174], Schistosome eggs[175], Cholera toxin subunit B[176,177]
Others	Galectin-1[178], Curcumin[179], Catalposide[180], Follistatin[123], Phex gene[181], FTY720[182], Matrine[183]

Table 3 Pathogenesis of IBD models in oxazolone colitis

Pathogenic factors	
Categories	Factors (References)
T cells	NKT[19], CEACAM1[27], Major basic protein[184], MHC class Ⅱ transactivator[185]
Cytokines/chemokines	IL-4[13], IL-13[19,40], EBI3[42]
Transcription factors	Smad7[72], NF-κB[67,68]
Others	Glycolipid[154]
Regulatory factors	
Categories	Factors (References)
T cells	Regulatory T cells[28]
Cytokines/chemokines	TGF-β[13]
Receptors	PAR-1[60]
Others	Budesonide[186]

conserved minor T-cell subset with characteristic properties that help maintain the homeostasis of epithelial cells, by providing a barrier between the luminal bacterial contents and underlying immune cells[22]. A regulatory role has been shown for TCRγδ T cells in DSS-[23,24] and TNBS-induced colitis models[25,26].

In addition to these populations, carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1; also known as CD66a) is a cell surface molecule that has been proposed to negatively regulate T cell function, and is associated with the regulation of T-bet-mediated Th1 cytokine signaling in TNBS- and oxazolone-induced colitis models[27].

Finally, regulatory T cells express the antigen non-specific suppressor factors transforming growth factor-β (TGF-β) and IL-10. Boirivant *et al* have shown that TNP-CP feeding cross-protects mice from an inflammatory response to a different hapten, oxazolone. This protective effect is associated with the appearance of mononuclear cells that produce regulatory cytokines[28]. This phenomenon of cross-protection could be exploited in designing novel treatments for IBD, because it demonstrates that an orally-administered antigen can induce production of regulatory cells that are able to suppress inflammation induced by a different type of antigen.

Cytokines/chemokines/growth factors

TNBS injection results in a transmural infiltrative colitis associated with an IL-12-mediated Th1-immune response[8]. In most cases, a single dose of TNBS is administered at the starting point of the experiment. In subsequent studies of IL-12, it has been reported that mucosal TNF-α is necessary for the initiation and perpetuation of TNBS colitis, since TNF-α-deficient mice are resistant to TNBS, and the colitis is extremely severe in mice that over-express TNF-α[29]. This result suggests that TNFα acts as a proximal co-factor for IL-12 or IL-18 production. One possible mechanism of amelioration by anti-IL-12 antibody treatment is through the induction of Fas-mediated apoptosis of Th1 cells[30].

Watanabe and colleagues have shown that TNBS-induced colitis is mediated by macrophage-derived IL-18[31]. In fact, neutralization with anti-IL-18 antibody results in dramatic attenuation of mucosal inflammation, and the administration of TNBS fails to induce significant colitis in IL-18 knockout (KO) mice. These results have been confirmed by another group who have demonstrated that recombinant human IL-18 binding protein isoform (rhIL-18BPa) leads to a significant reduction in TNBS-induced colitis, by decreasing local TNF-α production[32]. Interestingly, IL-18 is also a primary mediator of the inflammation in DSS-induced colitis, while neutralization of IL-18 attenuates intestinal damage in that colitis model[33].

In Th1-mediated colitis, the use of agents that block IL-12 secretion or activity provides the most direct approach for attenuating inflammation because IL-12 is critical for regulation of differentiation and activation of Th1 cells[8,30]. It has been demonstrated that IL-12p40 KO mice develop severe TNBS-induced colitis. Moreover, administration of IL-12p40 neutralizing antibody increases pathology in IL-12p35 KO mice, which suggests that IL-12p40, in contrast

to IL-12p70, exerts the major regulatory function in TNBS-induced colitis[34]. However, IL-12p40 forms heterodimers, not only with IL-12p35 (IL12p35p40; IL-12p70), but also with IL-23p19 (IL-23p19p40); a finding that raises the possibility that activity previously ascribed to IL-12 may be attributable to IL-23. Recently, it has been revealed that IL-23 is a key effector cytokine in the immune system of the intestine[35]. IL-23 specifically expands a pathogenic population of $CD4^+$ T cells called Th-17 cells, which produce IL-17A, IL-17F, IL-6 and TNF-α[36,37]. Indeed, IL-17R KO mice are protected against TNBS-induced colitis[38]. By contrast, Becker *et al*[39] have reported that IL-23 cross-regulates IL-12 production in T-cell-mediated TNBS colitis, since mice lacking the p19 subunit of IL-23 are highly susceptible to TNBS-induced colitis, and inhibition of IL-12p40 rescues IL-23p19 KO mice from lethal disease. These discrepancies regarding the role of IL-23 may result from different experimental models; therefore, further characterization should help in developing new therapeutic treatments for patients.

As for the Th2-type responses, oxazolone colitis is associated with increased production of IL-4/IL-5, and is prevented by the systemic co-administration of anti-IL-4 antibody[13]. Heller *et al*[19] have shown that IL-13, mainly produced by NK T cells, is a significant pathogenic factor in this model, since its neutralization by the decoy receptor IL-13Rα2-Fc prevents disease. As well, IL-13 induces TGF-β1, generally considered to be an anti-inflammatory cytokine, through IL-13Rα2 in oxazolone-induced colitis, and prevention of IL-13Rα2 expression leads to the marked down-regulation of TGF-β1 production and collagen deposition in bleomycin-induced lung fibrosis, during prolonged inflammation[40].

As an IL-12p40-related protein, it has been reported that Epstein-Barr virus-induced gene 3 (EBI3) dimerizes with a novel p28 subunit (which has homology to IL-12p35) to form the cytokine IL-27[41]. IL-27 has been shown to function as a proliferation factor for naïve, but not memory, $CD4^+$ T cells, and to synergize with IL-12 to stimulate IFN-γ production[41]. That EBI3 KO mice have been found to be resistant to oxazolone-induced colitis suggests that this molecule plays a crucial role in the induction of Th2-type immune responses[42].

Several families of growth factors regulate a wide spectrum of processes integral to IBD; including protection of the intestinal mucosa and activation, as well as regulation of the intestinal immune system. These factors mediate mucosal repair, restitution, remodeling and resolution of inflammation following tissue damage[43]. It is now widely accepted that TGF-β has an important function in regulating inflammation and tissue repair. Fuss *et al*[44] have elegantly demonstrated the relationship between TGF-β and IL-10 in the regulation of Th1-mediated inflammation in TNBS-induced colitis, by performing a study in which mice were fed a haptenated colonic protein and then administered either anti-TGF-β or anti-IL-10 antibody, at the time of subsequent rectal administration of TNBS. Anti-TGF-β antibody administration prevents TGF-β secretion, but leaves IL-10 secretion intact, whereas anti-IL-10 antibody administration inhibits both TGF-β and IL-10 secretion. Their data suggest that TGF-β alone is the primary mediator of counter-regulatory Th1-type mucosal inflammation, and that IL-10 is necessary as a secondary factor that facilitates TGF-β production, but does not act as a suppressor cytokine by itself. Interestingly, Kitani *et al*[45] have shown that single intranasal administration of DNA encoding active TGF-β prevents the development of Th1-mediated TNBS colitis. This study shows that following treatment, TGF-β-producing T cells and macrophages are found in the LP and spleen, in which they hypothetically act to prevent induction of TNBS colitis. Therapeutic strategies involving TGF-β-encoding DNA may provide beneficial effects in treating intestinal inflammation.

The role of TGF-α in the small intestine and colon has not been studied as extensively as it has been in the gastric mucosa. In DSS colitis, TGF-α is a mediator of protection and/or healing in the colon, which is demonstrated by the absence of disease in TGF-α-KO mice[46].

Hepatocyte growth factor (HGF) may be a critical regulatory factor in IBD since HGF activator-KO mice are unable to survive after DSS or acetic acid-induced colitis[47]. HGF promotes migration of gastrointestinal epithelial cells and accelerates wound repair by mucosal cells. The importance of HGF has been confirmed by the intrarectal administration of HGF-expressing adenovirus in TNBS-treated mice, which leads to significant improvements in mucosal damage[48]. The same group has also demonstrated the therapeutic effects of naked gene therapy of HGF in the DSS-induced colitis model[49]. Taking these results together, HGF gene delivery may be very useful as a therapeutic strategy for human IBD.

As well as HGF, basic fibroblast growth factor (bFGF or FGF-2) also improves mucosal damage by enhancing epithelial cell restitution and proliferation in the gastrointestinal tract[50]. In fact, rectal administration of human recombinant bFGF (hrbFGF) ameliorates DSS-induced colitis by significantly reducing the gene expression level of TNF-α[51]. Not only DSS-, but also TNBS-induced colitis is improved by the administration of hrbFGF, which not only enhances survival rate, but also up-regulates levels of cyclooxygenase (COX)-2, TGF-β, intestinal trefoil factor (ITF), and vascular endothelial growth factor (VEGF) in the colon[51].

Lastly, the trefoil factor family is comprised of three peptides; trefoil factor family 1 (TFF1), spasmolytic polypeptide (SP also known TFF2), and ITF (also known as TFF3). TFF2 is a low-molecular-weight protein that is up-regulated in gastric tissues infected with *Helicobacter* or affected by other inflammatory conditions[52]. TFF2 KO mice are susceptible to DSS-induced colitis, with prolonged colonic hemorrhage and persistent weight loss[53]. The importance of ITF in the modulation of inflammation, wound healing, and protection of the intestinal mucosa is supported by experiments in ITF KO mice, which have shown increased susceptibility and delayed wound healing during DSS- and acetic acid-induced colitis[54].

Receptors

TNF-α plays a central role in the pathology of Th1-mediated colitis such as CD; however, the role of its receptors, TNF receptor-type I (TNFR1) and -type Ⅱ

(TNFR2) in mediating pathology has not been fully explored. TNFR2 expression and signal transducer and activator of transcription (STAT) 3 activation in colonic epithelial cells (CECs) are markedly up-regulated during the recovery phase of DSS-induced acute colitis[55]. Recently, it has been reported that TNFR1 KO mice lose more weight and have increased mortality compared with wild-type mice, while TNFR2 KO mice lose less weight and have an improved survival rate compared to wild-type mice in TNBS-induced colitis. These results suggest that TNF-α signaling through TNFR1, but not TNFR2, is protective in mouse models of IBD[56].

As for Th1-type responses, CD40L-CD40 interaction is crucial for the priming of Th1 cells *via* the stimulation of IL-12 secretion by antigen-presenting cells (APC) in TNBS-induced colitis. The administration of anti-CD40L antibody prevents IFN-γ production and TNBS-induced colitis, which suggests that the Th1 response may be mediated by CD40L-CD40 interactions[57,58].

Recent studies have demonstrated that the proteinase-activated receptors (PARs), a family of G protein-coupled receptors activated by serine proteinases, have an important anti-inflammatory role in the colon. PAR-1 and -2 are highly expressed in CECs and neuronal elements, and are involved in regulating secretion by the epithelial cells of salivary glands, stomach, pancreas and the intestine[59]. Intracolonic administration of PAR-1 agonist in oxazolone-treated mice efficiently inhibits colitis[59]. By contrast, the inflammatory responses in PAR1 KO or PAR-1 antagonist-treated mice are exacerbated in oxazolone-induced colitis[60]. As well, PAR-2 activation prevents the development of TNBS-induced colitis[61].

Finally, the glucocorticoid-induced TNFR (GITR)-related gene is a member of the TNFR superfamily that is constitutively expressed at high levels on $CD4^+$ $CD25^+$ regulatory T cells, and at low levels on unstimulated T cells, B cells and macrophages[62]. GITR signalling in $CD4^+$ T cells is involved in the development and progression of colitis[63], while deletion of GITR protects against TNBS-induced colitis by reducing innate immune responses and effector T-cell activity[64].

Transcription factors

Nuclear factor (NF)-κB is the key transcription factor for pro-inflammatory responses, and is thought to be important in the initiation and progression of both human IBD and animal models of colitis[65,66]. Disease activity in mice with TNBS-induced colitis is inhibited by antisense oligonucleotides that inhibit the p65 subunit of NF-κB, which suggests a critical role for NF-κB in mediating inflammatory responses[65]. Attempts to control mucosal inflammation by the use of agents that block the NF-κB pathway have had some success in murine models. For example, it has been shown that administration of NF-κB decoy oligodeoxynucleotides (decoy ODNs) encapsulated in a viral envelope prevents the development of TNBS- and oxazolone-induced colitis by inhibiting production of IL-23/IL-17[67]. De Vry *et al* have used a chemically modified, non-viral NF-κB decoy and have shown that the NF-κB decoy ameliorates disease severity in TNBS-, DSS- and oxazolone- induced colitis. These studies suggest that NF-κB decoy ODNs are effective in attenuating Th1- as well as Th2-mediated colitis, and this would be a potentially useful therapeutic strategy for human IBD[68]. In addition to NF-κB, mitogen-activated protein kinase (MAPK) p38 is also a crucial mediator of inflammation. Inhibition of NF-κB and MAPK p38 by SB203580 is able to attenuate the inflammatory response in TNBS-induced colitis models[69,70].

By contrast, TGF-β1 functions as a negative regulator of T-cell immune responses, signaling target cells through the Smad family of proteins. Smad7, an inhibitor of TGF-β1 signaling, is over-expressed in the intestinal mucosa and purified mucosal T cells isolated from patients with IBD[71]. Oral administration of antisense oligonucleotide of Smad7 also ameliorates inflammation in TNBS- and oxazolone-induced colitis, by restoring TGF-β1 signaling *via* Smad3[72].

It has been demonstrated that cytokines exert their biological functions through Janus tyrosine kinases and STAT transcription factors. An experiment blocking the IL-6 receptor has demonstrated that IL-6 plays an important role in the development of Th1-mediated TNBS-induced colitis by activating the STAT3 signaling pathway[73]. Indeed, STAT3 was most strongly tyrosine-phosphorylated in human UC and CD patients and in DSS-induced colitis in mice[74]. These results suggest that the IL-6/STAT3 pathway plays a crucial role in the development and perpetuation of DSS-induced colitis.

Lastly, peroxisome proliferator-activated receptor γ (PPARγ) is a lipid-activated transcription factor, and PPARγ heterozygous mice are highly susceptible to TNBS-[75] and DSS-induced colitis[76]. It has also been reported that mice with a targeted disruption of PPARγ in macrophages display an increased susceptibility to DSS-induced colitis[77]. Therefore, activation of PPARγ may potentially protect against human IBD.

Adhesion molecules

Trafficking, activation and retention of leukocytes within inflamed tissues are mediated by several classes of specialized adhesion glycoproteins[78]. Collagens represent the most abundant extracellular matrix protein, and the major cell surface receptors for collagens are integrins[78,79]. The collagen-binding integrin α1β1 mediates inflammation in TNBS-[80] and DSS-induced colitis[81], which suggests the importance of α1β1-mediated adhesive leukocyte/matrix interactions in regulating mucosal inflammatory responses. Leukocyte β2 integrins are heterodimeric adhesion molecules consisting of a common β subunit (CD18) and different α subunits (CD11a-d)[82]. In DSS-induced colitis, leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18) seems to have a pathogenic role, whereas Integrin alpha M (Mac-1α, CD11b/CD18) serves in a regulatory capacity[83]. Much attention has been focused on the role of α4 integrin in IBD, but it has recently been reported that neutralization therapy may result in undesirable complications such as multifocal leukoencephalopathy[84].

Toll-like receptors (TLRs) and their ligands

It is widely suspected that IBD arises from a dysregulated mucosal immune response to luminal bacteria. TLRs,

which are pattern-recognition receptors expressed by both immune and non-immune cells, play a pivotal role in host/microbial interactions and have two distinct functions-protection from infection and control of tissue homeostasis, depending on the recognition of pathogens or commensals[85-88]. TLRs send intracellular signals in response to intestinal commensal or pathogenic microbes that contain or release conserved molecular patterns, such as LPS, bacterial lipoprotein, bacterial cytosine-guanosine dinucleotide (CpG) DNA, and bacterial flagellin. Activation of TLRs results in the activation of the innate and/or adaptive immune response[85]. In this context, TLRs play an important role in the maintenance of intestinal homeostasis. TLR4 recognizes LPS, and transduces a proinflammatory signal through the adapter molecule myeloid differentiation marker 88 (MyD88)[86]. DSS treatment of TLR4 KO and MyD88 KO mice has been shown to induce earlier and more severe colitis compared to that in wild-type mice, which suggests that TLR4 signaling through MyD88 is an important suppressor of the inflammatory response to chemical injury[87].

Bacterial flagellin specifically stimulates TLR5 and activates MAPK and NF-κB-related signaling pathways, which leads to the production of macrophage inflammatory protein 3α (MIP3α) and IL-8[89]. Flagellin exposure exacerbates inflammation in DSS-induced colitis, but not in the intact colon[88]. By contrast, a TLR2 specific agonist, peptidoglycan or lipoteichoic acid, does not cause any inflammatory response[90].

Lastly, TLR9 is critical for the recognition of the CpG motif of bacterial DNA[91]. DSS-induced colitis is less severe in TLR-9 KO mice[92], and treatment of mice with an adenovirus expressing CpG-ODN that is known to block CpG effects results in significant amelioration of DSS-induced colitis[92], which indicates that ODN inhibition of the immune-stimulating properties of bacterial DNA may offer a novel and specific tool for the treatment of IBD.

Enzymes

Although intestinal epithelial cells constitutively express COX-1, COX-2 is induced only during inflammatory conditions. Enzymatic activity of these COX isoforms produces prostaglandins (PGs) that have proinflammatory roles mediating fever, hyperalgesia, vascular permeability and edema. However, PGs also have a protective role against gastrointestinal injury[93]. The linkage between COX-2 and PGE2 for protection against colitis has been highlighted in various studies. For example, COX-2 KO mice are more susceptible to DSS-induced colitis, which correlates with their inability to produce PGE2[94]. Kabashima *et al*[95] have used mice deficient in prostaglandin receptor EP4 and examined the roles of prostanoids in DSS-induced colitis; their mice developed severe colitis, which suggests that EP4 maintains intestinal homeostasis by keeping mucosal integrity and down-regulating immune responses. It has also been shown that COX-2-derived PGE2 is important in TLR4-related mucosal repair[96], and that COX-2 has a protective effect against acetic-acid-induced colitis[97,98]. These results suggest that COX-2 has a pivotal role in the maintenance of mucosal homeostasis. However, there is controversy about whether COX-2 inhibitors worsen symptoms of human IBD[99].

Through the use of DNA microarray analysis, our group has demonstrated that several detoxification-associated molecules, which contribute to the prevention of inflammation by regulating physiological balance under normal conditions, are highly down-regulated in CECs in chronic colitis[100]. Among the up-regulated detoxification-associated molecules, carbonic anhydrase (CAR)-Ⅳ is an important enzyme involved in the suppression of acidification, by regulating mucosal bicarbonate concentration[101]. Unexpectedly, inhibition of CAR-Ⅳ suppresses the severity of DSS-induced colitis but enhances CEC proliferation, which raises the possibility that CAR-Ⅳ may have a pathogenic role under inflammatory conditions. Microarray analysis also identifies chitinase 3-like-1 (CHI3L1) as being specifically up-regulated in inflamed mucosa[102]. The expression of CHI3L1 protein is detectable in LP and CECs in several murine colitis models, and also in IBD patients, but is absent in normal controls. Anti-CHI3L1 antibody administration significantly ameliorates DSS-induced colitis, which suggests that inhibition of CHI3L1 activity may be a novel therapeutic approach for IBD. Our group is currently investigating this possibility by utilizing murine models of chronic colitis.

As well, IL-1β-converting enzyme (ICE), also known as caspase-1, is an intracellular protease that cleaves the precursors of IL-1β and IL-18 into active cytokines[103,104]. ICE deficiency results in protection from DSS-induced colitis, accompanied by the reduced release of the proinflammatory cytokines IL-18, IL-1β and IFN-γ[105].

Lastly, recent studies have identified Vanin-1 as being involved in the regulation of innate immunity. Vanin-1 is an epithelial ectoenzyme with pantetheinase activity, which is involved in the metabolic pathway of pantothenate (vitamin B5), and provides cysteamine to tissues[106]. Vanin-1 deficiency protects from TNBS-induced colitis. Additionally, by antagonizing PPARγ, Vanin-1 promotes the production of inflammatory mediators by intestinal epithelial cells[107]. This study suggests that Vanin-1 is an epithelial sensor of stress that exerts control over innate immune responses in tissues. As such, it has been proposed as a potential new therapeutic target for IBD.

Hormones

It has been demonstrated that adipose tissue secretes a variety of biologically active molecules[108]. Adiponectin (APN) is an adipose tissue-derived hormone and is considered to be a member of the expanding family of adipokines[109]. APN has a protective role against DSS-induced murine colitis, but not TNBS-induced disease[110], by inhibiting the production of chemokines such as monocyte chemoattractant protein-1 and MIP-2 in CECs, and the subsequent inflammatory response. However, a proinflammatory role for APN in synovial fibroblasts[111] and CECs[112] has recently been suggested. APN exerts proinflammatory activity in the colon by producing proinflammatory cytokines and inhibiting the bioactivity of protective growth factors such as bFGF and heparin-

binding epidermal growth factor. It is interesting to note that APN KO mice are highly protected from both DSS- and TNBS-induced colitis[112].

Finally, leptin, a regulator of food intake and energy expenditure, can also modulate immune and inflammatory responses. Leptin-deficient (ob/ob) mice exhibit less severe colitis compared to wild-type mice in DSS and TNBS models, while replacement of leptin in ob/ob mice converts disease resistance to susceptibility, which indicates that leptin deficiency accounts for the resistance to acute DSS- and TNBS-induced colitis[113]. It has also been shown that phosphorylation of STAT3 and induction of COX-2 are absent in the colon of ob/ob mice[113]. Therefore, leptin represents a functional link between the endocrine and immune systems.

CONCLUSION

Dysregulated immune responses initiated by microbial-host interactions contribute to the development and perpetuation of both murine colitis and, most likely, to human IBD. In this process, intestinal epithelial cells play important roles linking innate and acquired immune responses. In this review, we have focused primarily on the role of functionally distinct factors in the pathogenesis of chemically-induced models of intestinal inflammation during acute, recovery and chronic phases. The increasing clinical use of biological therapy in human IBD illustrates the potential benefits that may be derived from molecular analysis of immunopathogenesis. However, the long-term effects of such therapy have still not been determined, and concerns regarding potentially increased risks of infection or tumor development have been raised, given the essential roles of innate and acquired immunity in host defense. In this respect, topical treatment would have the advantage of selectively targeting local immune responses while sparing systemic immune protective mechanisms. Therefore, we need to find agents that have more targeted effects or take advantage of local delivery systems that target diseased lesions, such as is seen with oligonucleotide-based therapeutics. The different animal models provide an easy means to study factors involved in pathogenesis and to test new therapeutic agents for human IBD.

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Genetic factors associated with the development of inflammatory bowel disease

Jesus K Yamamoto-Furusho

Jesus K Yamamoto-Furusho, Inflammatory Bowel Disease Clinic, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición, Vasco de Quiroga 15, Colonia Sección XVI, Tlalpan, C.P. 14000, México, D.F., México
Correspondence to: Jesús K Yamamoto-Furusho, MD, PhD, MSc, Head of Inflammatory Bowel Disease Clinic, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga 15, Col. Sección XVI, Tlalpan, México, D.F., Mexico. kazuofurusho@hotmail.com
Telephone: +52-55-55733418 Fax: +52-55-56550942
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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are complex polygenic disorders, characterized by several genes together with environmental factors contributing to the development of inflammatory bowel disease (IBD). Recent advances in research on genetic susceptibility have allowed the identification of diverse genes at different levels: (1) Innate immunity; (2) Antigen presentation molecules; (3) Epithelial integrity; (4) Drug transporter; (5) Cell adhesion. The application of genetic testing into clinical practice is close and all genetic markers may have several clinical implications: prediction of disease phenotype, molecular classification, prevention of complications, and prognosis.

Key words: Genetic; Susceptibility; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing inflammatory bowel diseases (IBD) of unknown etiology. CD and UC are complex polygenic disorders, characterized by several genes together with environmental factors contributing to the development of IBD.

A variety of epidemiological and clinical data suggest that genetic factors are intimately involved in the pathogenesis of IBD including familial aggregation pattern of disease with a much higher disease frequency in first degree relatives of affected individuals compared with the general population. Twin studies provide the argument for a genetic basis for IBD, with a much higher rate of disease concordance observed in monozygotic than in dizygotic twins and wide variations in the incidence and prevalence of IBD among different populations[1].

IBD is now considered a non-Mendelian polygenic disorder with important environmental interactions (e.g., microbial factors, smoking).

There are two main approaches to identifying genes in complex multifactorial diseases: the positional cloning approach based on linkage studies, and the candidate gene approach based on association studies. Linkage analysis studies the cosegregation of the disease with a marker within the families. Linkage analysis allows scanning of the whole genome. Eleven of these total genome scans have been undertaken in IBD, resulting in a number of susceptibility regions on chromosomes 1, 3, 4, 5, 6, 7, 10, 12, 14, 16, 19 and X[2]. According to their initial date of reporting and independent confirmations, the regions on chromosomes 16q, 12, 6, 14, 5, 19, 1, 16p and 10 have been renamed IBD 1 to IBD 9, respectively. However, new genes have been reported recently. All susceptibility genes discovered can be categorized into different levels of susceptibility: (1) Innate immunity; (2) Human leucocyte antigen (HLA) molecules; (3) Epithelial integrity (4); Drug transporter; and (5) Cell adhesion.

INNATE IMMUNITY

Understanding of innate immunity has progressed enormously with the discovery of many microbial sensors called pattern recognition receptors (PRRs). The toll-like receptor (TLR) and nucleotide oligomerization domain (NOD) receptor families of PRRs appear to play essential roles in mucosal homeostasis and their alterations contribute to the pathogenesis of IBD.

NOD2 is expressed constitutively in macrophages, neutrophils and dendritic cells[3], as well as in Paneth cells and is induced in epithelial cells[4]. NOD2 is a cytoplasmic protein that serves as a microbial sensor, and its leucine-rich repeat (LRR) domain is required for recognition of muramyl dipeptide (MDP), a fragment of peptidoglycan

present in bacterial cell walls. The ligand MDP ultimately leads to activation of the transcription factor nuclear factor kappa B (NF-κB), and induction of proinflammatory cytokines[5,6]. Specific mutations of the NOD2 gene have been definitively associated with increased susceptibility to ileal Crohn's disease in Western (but not Asian) populations: Arg702Trp, Gly908Arg, and leu1007fsinsC (a frame shift mutation that truncates the carboxy terminal 33 aminoacids)[7,8]. Heterozygous carriage of the risk alleles confers a 2-4 fold increased risk, and homozygotes or compound heterozygotes have a 20-40 fold increased risk[9]. More than 90% of all CD- associated mutations are located in the LRR domain, suggesting that these may affect the function of NOD2 with respect to bacterial recognition and signaling.

NOD1/ CARD4 (Caspase Recruitment Domain 4) plays a role in colonic epithelial defense against *E. coli* and *S. flexeneri* and mediates NF-κB activation[3,10]. Recently, genetic variants of NOD1 have been shown to be associated with disease susceptibility. In a recent study of 556 patients with IBD (294 CD and 252 UC), an association between the variant (rs695857) in nucleotides 30, 258 and 950 of NOD1 and the development of IBD was found. Another variant known as rs2907748 in nucleotides 30, 246 and 263 was also associated with the presence of UC and CD and even with the early onset of the disease (< 25 years of age)[11].

TLRs are abundantly expressed on the surface of monocytes, macrophages, dendritic and epithelial cells. Alterations of TLR3 and TLR4 expression by intestinal epithelial cells have been described in IBD[12], suggesting that there is differential expression of TLR family members. Two common polymorphisms of TLR4 (Asp299Gly and Thr399Ile) have been described in humans. Asp299Gly has been associated with reduced responsiveness following lipopolysaccharide stimulation[13]. These polymorphisms have been associated with the development of CD and UC in Caucasian populations[14-16]. Pierik *et al*[17] showed that TLR1 R80T and TLR2 R753G polymorphisms were associated with pancolitis in UC patients, while a negative association was observed between TLR6 S249P and proctitis in patients with UC. These results suggest that TLR2 and its co-receptors TLR1 and TLR6 are involved in the initial immune response to bacteria in the pathogenesis of IBD.

ANTIGEN PRESENTATION MOLECULES

The major histocompatibility complex (MHC) region is the region studied most extensively. Human leucocyte antigen (HLA) class Ⅱ molecules present partially digested antigen to the T-cell receptor and play a central role in the immune response. The mechanism by which classical HLA class Ⅱ genes exert their influence in IBD is unknown. Different HLA molecules may bind preferentially to different peptides, or bind the same peptide with varying affinity. In IBD, cross reactivity (known as molecular mimicry) may exist between the peptides derived from bacterial luminal flora and from self antigens present in the gut. This may lead to the generation of auto reactive T cells which contribute to disease pathogenesis. HLA-DRB1 is the most extensively studied gene in IBD. In a meta-analysis made by Stokkers *et al*[18], positive associations between UC and HLA-DR2, HLA-DRB1*1502 (OR = 3.74, CI: 2.2-6.38), HLA-DR9 (OR = 1.54, CI: 1.06-2.24) and HLA-DRB1*0103 (OR = 3.42, CI: 1.52-3.69) were found; a negative association was found with HLA-DR4 (OR = 0.54, CI: 0.43-0.68). Another study found that HLA-DRB1*0103 allele was associated with UC and its severe manifestations such as colectomy and pancolitis (P = 0.003, OR = 3.6, CI 95%: 1.46-8.9), while HLA-DRB1*15 allele was only associated with pancolitis in patients with UC (P = 0.001, OR = 8.5)[19].

On the other hand, HLA class Ⅲ genes have been associated with IBD. Several studies have shown the role of tumor necrosis factor α (TNF α) polymorphisms in IBD. There are specific genetic polymorphisms involving TNFα that influence the amount of cytokine produced. Bouma *et al*[20] reported an association between the polymorphism of *TNF*α gene promoter region at -308 position and UC, and this finding was confirmed by other studies[21, 22].

EPITHELIAL INTEGRITY

The organic cation transporter (OCTN) is a family of transporter proteins for organic cations, and may also transport carnitine, an essential cofactor of the metabolism of lipids. Carnitine is involved in the transport of long-chain fatty acids into the mitochondria. There is evidence that inhibition of fatty acid oxidation in the epithelium of the colonic mucosa is associated with the development of UC. There are two subtypes of this gene, OCTN1 and OCTN2, and some mutations have been reported in them: SLC22A4 1672C/T for OCTN1 and SLC22A5-207G/C for OCTN2, which are associated with the development of CD. The presence or combination of these mutations constitutes TC haplotype, which is associated with ileal, colonic and perianal affection and onset and the need of surgical treatment in CD[23, 24].

DLG5 (Drosophila long disc homologue 5) gene is a member of the membrane associated guanylate kinase gene family which encodes cell scaffolding proteins and seems to play a role in the maintenance of intestinal epithelial cells, and its mutations have been involved in a rise in intestinal permeability[25]. DLG5 is a widely expressed protein found in many tissues such as the placenta, small bowel, colon, heart, skeletal muscle, liver and pancreas. It is important in signal transduction and epithelial cell integrity. Four haplotypes have been identified, but only D haplotypes were associated with UC and CD in a European cohort[26]. Another variant of this gene (rs37462) was found in Japanese people with CD[27]. The haplotype characterized by the haplotype-tagging single nucleotide polymorphisms (SNP) G113A called haplotype D, was found substantially over-transmitted in patients with IBD.

DRUG TRANSPORTER

The multidrug-resistance (MDR-1) gene encodes the drug efflux pump P-glycoprotein 170 (Pgp-170). Various polymorphisms have been identified within MDR-1: a

mutation C3435T in exon 26 and a mutation G2677T in exon 21 have been correlated with altered Pgp expression and function in humans. Overexpression of MDR-1 leads to an increased efflux of drugs and decreased cytoplasmic drug concentrations. Several drugs, including glucocorticoids, are known Pgp-170 substrates. Farrell *et al*[28] showed that MDR was significantly elevated in CD and UC patients who required bowel resection and proctocolectomy after failed medical therapy. Variant C3435T was related to the presence of pancolitis in patients with UC in Scotland[29]. However, the frequency of SNPs is low and is different among populations, with the exception of three SNPs in exon 12 (C1236T), exon 20 (G2677T/A) and exon 26 (C3435T), and some of them are correlated with different diseases and clinical characteristics[30].

CELL ADHESION

Cell surface adhesion molecules conveying leukocyte-endothelial interactions, govern homing of activated inflammatory cells into gut. Extravasation and migration into the site of inflammation are mediated by integrins and selectins, and these molecules are increased in IBD patients. There are targeting therapies against adhesion molecules in clinical trials to date including natalizumab (integrin α4 subunit) and MLN-02 (selective adhesion molecule blocker for integrin $\alpha 4\beta 7$). In Japanese patients with IBD, the intercellular adhesion molecule-1 (ICAM-1) K469 allele is associated with CD and UC[31].

CONCLUSION

There are increasing numbers of genetic markers associated with the development of IBD at different levels: innate immunity, antigen presentation, epithelial integrity, drug transporter and cell adhesion that contribute, in genetic susceptibility, to the development of IBD in conjunction with environmental and immunological factors.

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TOPIC HIGHLIGHT

Jesus K Yamamoto-Furusho, Dr, *Series Editor*

Pouchitis

En-Da Yu, Zhuo Shao, Bo Shen

En-Da Yu, Department of Colorectal Surgery, Shanghai Changhai Hospital, Shanghai 200438, China
Zhuo Shao, Department of Biliary Surgery, Eastern Hepatobiliary Hospital, Shanghai 200438, China
Bo Shen, Digestive Disease Center, Cleveland Clinic, Cleveland, OH 44195, United States
Supported by a grant from National Institutes of Health, No.R03 DK 067275 and a grant from Broad Medical Research Program of Broad Foundation
Correspondence to: Bo Shen, MD, Department of Gastroenterology /Hepatology-Desk A30, Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44195, United States. shenb@ccf.org
Telephone: +1-216-4449252 Fax: +1-216-4446305
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Abstract

While restorative proctocolectomy with ileal pouch-anal anastomosis has significantly improved the quality of life in patients with underlying ulcerative colitis who require surgery, complications can occur. Pouchitis as the most common long-term complication represents a spectrum of disease processes ranging from acute, antibiotic-responsive type to chronic antibiotic-refractory entity. Accurate diagnosis using a combined assessment of symptoms, endoscopy and histology and the stratification of clinical phenotypes is important for treatment and prognosis the disease. The majority of patients respond favorably to antibiotic therapy. However, management of chronic antibiotic-refractory pouchitis remains a challenge.

Key words: Classification; Complication; Ileal pouch; Inflammatory bowel disease; Restorative proctocolectomy

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INTRODUCTION

Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) has become a part of standard surgical treatment for patients with ulcerative colitis (UC) or familial adenomatous polyposis (FAP). Despite advances in medical therapy, approximately 30% of patients with UC eventually require total proctocolectomy[1]. Restorative proctocolectomy with IPAA has the following advantages: (1) gastrointestinal continuity is reestablished with IPAA, (2) the procedure helps improve symptoms of patients and health-related quality of life, (3) the majority of patients with IPAA can avoid UC-related medications, particularly immunomodulators and biological agents and their associated potential adverse effects, and (4) IPAA with proctocolectomy substantially reduces the risk for dysplasia or cancer. However, adverse outcomes or complications often occur after surgery. Common long-term inflammatory and functional complications of restorative proctocolectomy are pouchitis, Crohn's disease (CD) of the pouch, cuffitis (inflammation in the rectal muscular cuff), and irritable pouch syndrome (IPS). Pouchitis likely represents a spectrum of disease processes ranging from acute antibiotic-responsive entity to chronic antibiotic-refractory type. Accurate diagnosis and classification of pouchitis are important for its proper management and prognosis.

INCIDENCE AND PREVALENCE

Pouchitis, a nonspecific inflammatory condition at the ileal pouch reservoir, is the most common long-term complication in patients with IPAA which significantly affects patients' quality of life[2]. Reported cumulative frequency rates of pouchitis 10-11 years after IPAA surgery range from 23% to 46%[3,4]. It is estimated that approximately 50% of patients who have undergone IPAA surgery for UC will develop at least one episode of pouchitis[5]. The estimated incidence within 12 mo after ileostomy was as high as 40% in a European study[6]. The discrepancy in the reported cumulative frequencies from different institutions likely results from diagnostic criteria used (e.g., diagnosis made based on symptom assessment alone or on a combined assessment of symptoms, endoscopy, with or without histology), intensity of follow-up with pouch endoscopy, and inclusion or exclusion of other inflammatory or functional disorders of the pouch and surgery related conditions (such as abscess, fistula, and sinus of the pouch).

ETIOLOGY AND PATHOGENESIS

Pouchitis almost exclusively occurs in patients with underlying UC and is rarely seen in patients with FAP[7,8]. Although the etiology and pathogenesis of pouchitis are

not entirely clear, bulk of evidence points towards an abnormal mucosal immune response (innate and adaptive) to altered microflora in the pouch leading to acute and/or chronic inflammation[6,9,10,11,12,13]. The prevailing theory holds that pouchitis results from an overgrowth of certain commensal bacteria[9,13,14,15]. Pouchitis only develops after ileostomy, i.e., the pouch mucosa starts to expose fecal stream. Manipulation of microflora with antibiotic or probiotic therapy resulting in improvement in patients with pouchitis provides additional evidence of involvement of microflora in the pathogenesis of pouchitis.

Immune mechanisms for pouchitis have been extensively studied in a similar fashion to that for inflammatory bowel disease. There are overlaps in tissue cytokine profiles between pouchitis and UC. However, pouchitis is not simply a duplication of the disease process seen in UC. The role of T-cell-mediated intestinal immunity in the pathogenesis of pouchitis is not entirely clear and is likely secondary to alterations in pouch microflora. Alterations in the macrophage and T cell subpopulations have been postulated in the process of pouchitis[16,17,18]. Increased T-cell activation and proliferation have been demonstrated in pouchitis, as evidenced by an increased expression in activation markers, such as CD25, CD30, and CD27[18]. As a result of activation of T cells and other immune cells, production of cytokines is up-regulated. Abnormal cytokine profiles have been reported in pouchitis including a deregulated production of proinflammatory and immunoregulatory cytokines[19]. Proinflammatory cytokines, such as TNF-α, are released at a great extent in the inflamed mucosa by macrophages and monocytes, leading to tissue injury, and are considered to be involved in pouchitis as a secondary pathophysiologic mechanism[19]. As in UC, the production of other inflammatory mediators including cytokines (such as IL-1β, IL-6, and IL-8)[20,21,22,30], cell adhesion molecules (such as E selectin and intercellular adhesion molecule-1)[23], platelet-activating factor[24], lipoxygenase products of arachidonic acids (such as leukotriene B4 and prostaglandin E2)[25], proinflammatory neuropeptides[26], macrophage inflammatory protein (MIP) 2α, matrix metalloproteinase (MMP)-1[21,27], MMP-2[21,27,28], MMP-9[28], MRP-14[21], and inducible nitric oxide[28], is also increased. Abnormalities in immunoregulatory cytokines such as IL-2, and interferon-γ[18,29], IL-4[29], and IL-10 are also seen in pouchitis. Imbalance between proinflammatory and immunoregulatory cytokines has been described in patients with pouchitis[30]. Abnormalities of T cells and other immune cells may not explain the whole mechanism of pouchitis. It is likely that such abnormalities are nonspecific and secondary in nature. Inconsistent results in the studies of immune cells and inflammatory mediators in pouchitis reflect the complexity in pathogenesis of the disease.

There are few published studies addressing the interplay between microflora and mucosal immune system in pouchitis. Exposure of peripheral blood and lamina propria lymphocytes *ex vivo* to sonicated flora from pouchitis induces more intense proliferation as compared with sonicates from healthy pouches. *In vitro* pretreatment of the sonicate preparation of pouch flora with metronidazole abolishes the stimulating ability[31]. Bacterial sonicates from a heterologous but healthy pouch do not stimulate lymphocyte proliferation[31]. The greater stimulatory effect of sonicates from pouchitis suggests that certain microflora may predominantly present in inflamed pouch mucosa and these microflora may be potentially pathogenic in activation of local mononuclear cells[31].

One of the most intriguing aspects of pouchitis is that it occurs almost exclusively in patients with underlying UC. Interestingly, there are similarities in terms of clinical presentations and immunological abnormalities between pouchitis and UC, suggesting that a subset of pouchitis may actually represent the recurrence of a UC-like disease in the ileal pouch. The theory of recurrent UC is supported by several lines of evidence. With the presence of stasis in the pouch, exposure to fecal contents and an increased microbial load could cause inflammatory changes leading to morphological alterations in the ileal pouch mucosa mimicking colon epithelia in UC[24]. Colonic metaplasia of the pouch mucosa seems to be a nonspecific adaptive response to the new luminal environment[24]. Colonic metaplasia characterized by villous blunting, crypt cell hyperplasia, and colon epithelium-specific antigens such as human tropomyosin 5, may increase the risk for pouchitis[32]. A similar alteration in mucin glycoproteins occurs in pouchitis as seen in UC[33]. It is possible that the altered glycoproteins are more susceptible to enzymatic degradation by bacteria, making the mucus barrier less resistant[34]. Additionally, some patients with pouchitis have the same extra-intestinal manifestations (such as arthralgia and primary sclerosing cholangitis or PSC) as those seen in patients with UC[35]. Smoking tends to have a protective effect against the development of pouchitis as it does against UC[36].

RISK FACTORS

Risk factors and potential predictors for pouchitis have been extensively studied. The implications of these studies include identification of etiopathogenetic factors, provision of strategies for modification of certain risk factors, and prediction of pouch outcome. Genetic polymorphisms such as those of IL-1 receptor antagonist[38,39,40] and NOD2/CARD15[40] may increase the risk for pouchitis. The reported risk factors for pouchitis also include non-carrier status of TNF allele 2[39], extensive UC[4,41,42], backwash ileitis[41], pre-proctocolectomy thrombocytosis[43], extra-intestinal manifestations, especially PSC[3,35,44,45], the presence of serum perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA)[46,47], being a non-smoker[36,42,48], and use of non-steroidal anti-inflammatory drugs (NSAID)[42,48]. In addition to p-ANCA, the presence of serologic markers, anti-*Saccharomyces cervesiae* antibodies to CD-related antigen from *Pseudomonas fluorescens* or outer membrane porin C of *Escherichia coli* in patients with pre-operative indeterminate colitis appears to be associated with persistent inflammation of the pouch after restorative proctocolectomy[49]. Acute and chronic pouchitis may have different risk factors[42].

It appears that few studies came up with the same risk factors. This inherent discrepancy among the studies may be contributed to the following factors: (1) small *vs* large

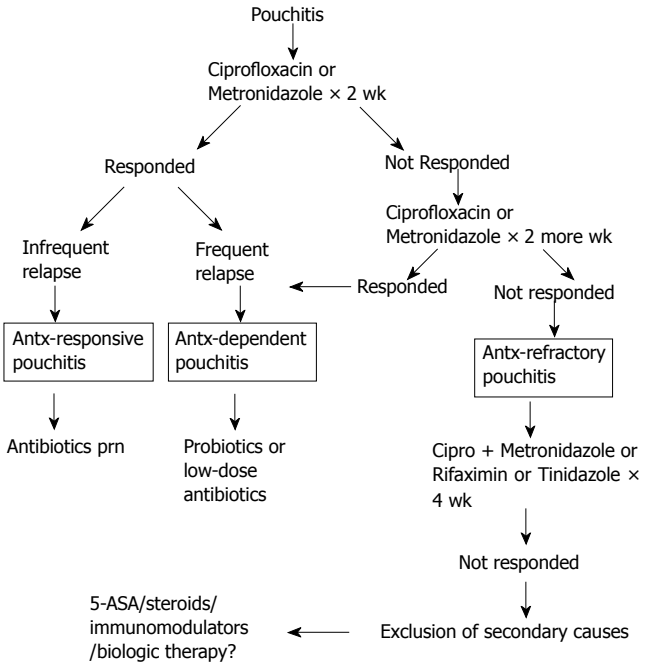

Figure 1 Classification and treatment algorithm.

sample sizes were analyzed, (2) the number of variables and outcomes was studied, (3) univariable analyses *vs* multivariable analyses were used, (4) diagnostic criteria for pouchitis were used, (5) pouchitis was stratified into acute *vs* chronic entities, and (6) type of controls was compared.

CLINICAL PRESENTATIONS

Patients with pouchitis have a wide range of clinical presentations, including increased stool frequency, urgency, tenesmus, incontinence, nocturnal seepage, abdominal cramping, and pelvic discomfort. While bloody bowel movements are uncommon in typical pouchitis, patients with IPAA with or without pouchitis can have iron deficiency anemia[50,51]. Patients with severe pouchitis occasionally present with fever, dehydration, malnutrition which may require hospitalization. Patients may have predominant extra-intestinal symptoms such as arthralgia and uveitis. These symptoms, however, are not specific and can present in disorders of the pouch other than pouchitis, such as cuffitis, CD of the pouch, proximal small bowel bacterial overgrowth, and IPS.

DIAGNOSTIC EVALUATIONS

Making diagnosis of pouchitis should not solely rely on presenting symptoms. The severity of symptoms does not necessarily correlate with the degree of endoscopic or histologic inflammation of the pouch[52,53]. A combined assessment of symptoms, endoscopic and histologic features is the key to making an accurate diagnosis and it is necessary to differentiate pouchitis from other inflammatory and non-inflammatory disorders of the pouch such as cuffitis, pouch stricture, pouch sinus, and IPS. There are no universally accepted diagnostic criteria for pouchitis. For clinical trials, the 18-point pouchitis disease activity index (PDAI) is most commonly used in the diagnosis of pouchitis and measurement of disease activity[54].

Pouch endoscopy yields valuable information on severity and extent of mucosal inflammation, presence or absence of concurrent ileitis or cuffitis, and structural abnormalities such as strictures, sinuses, and fistula openings. In addition, pouch endoscopy with segmental biopsy is the tool for dysplasia surveillance and can deliver effective therapy, including stricture dilation. Histopathology is invaluable for the detection of dysplasia, viral inclusion bodies of cytomegalovirus infection, granulomas, pyloric gland metaplasia, mucosal prolapse, and ischemic changes. It should be pointed out that villous blunting and an increased number of mononuclear cells in the lamina propria can be a part of "normal" adaptive changes of pouch mucosa to fecal stasis in the pouch which does not necessarily indicate pouchitis.

In cases of suspected complicated pouchitis, imaging studies such as contrasted pouchography, CT and MRI are typically used to assess the presence of mucosal and transmural disease activity within and around the pouch[55]. Wireless capsule endoscopy appears safe in patients with IPAA, which has been used for diagnostic evaluation in patients with chronic pouchitis[56] or anemia[57]. For patients with symptoms of dyschezia and feeling of incomplete evacuation, anal pouch manometry may be used to identify functional abnormalities such as paradoxical contractions.

CLINICAL CLASSIFICATION

Pouchitis likely represents a disease spectrum from acute, antibiotic-responsive type to chronic, antibiotic-refractory entity. From various perspectives, pouchitis can be categorized into: (1) idiopathic vs secondary based on etiology, (2) remission *vs* active based on disease status, (3) acute *vs* chronic based on disease duration, (4) infrequent episodes *vs* relapsing or continuous based on disease course, and (5) responsive *vs* refractory based on response to antibiotic therapy[58]. A subpopulation of patients has pouchitis associated with identifiable and modifiable causes (namely secondary pouchitis), such as *Clostridium difficile*[59,60] or cytomegalovirus[61,62] infection, and regular use of NSAID[63].

While the majority of patients with pouchitis respond favorably to antibiotic therapy particularly at initial stages of the disease, some patients develop pouchitis refractory to regular antibiotic treatment. This leads to another useful clinical classification based on the response to antibiotic therapy[64]. Analogous to the classification of UC according to the response to or dependency on corticosteroids, pouchitis can be classified as antibiotic-responsive, antibiotic-dependent, and antibiotic-refractory pouchitis[48,64] based on the manner of the patients' response to antibiotics (Figure 1).

TREATMENT

As the majority of patients develop acute pouchitis within the first year after IPAA[65], VSL#3® containing 4 strains of *Lactobacillus*, 3 *Bifidobacterium* species, and *Streptococcus*

salivarius subsp. *Thermophillus* was evaluated for the primary prophylaxis for the initial episode of pouchitis. Two of 20 patients (10%) treated with VSL#3® developed pouchitis within 12 mo after IPAA, while 8 of 20 patients (40%) experienced pouchitis in the placebo group during the same period of time[6].

The management and prognosis vary in different types of pouchitis (Figure 1). For antibiotic-responsive pouchitis, the first-line therapy includes a 14-d course of oral metronidazole (15-20 mg/kg per day) or ciprofloxacin (1000 mg/d)[66,67]. A randomized trial of ciprofloxacin and metronidazole showed that patients treated with ciprofloxacin experience significantly greater reductions in the PDAI scores and fewer adverse effects than those treated with metronidazole[67]. Other agents have been reported in open-labeled trials including tetracycline, clarithromycin, amoxicillin/clavulanic acid, doxycycline, rifaximin, and budesonide enemas[68], alicaforsen enemas, an anti-sense inhibitor of intercellular adhesion molecule-1[69], and AST-120, a highly adsorptive, porous, carbon microspheres[70].

Patients with antibiotic-dependent pouchitis often require long-term maintenance therapy with either antibiotics or probiotics to keep disease in remission. A randomized trial of VSL#3® at a dose of 6 g/d was conducted for the secondary prophylaxis for relapse of pouchitis, after remission was induced by oral ciprofloxacin (1000 mg/d) and rifaximin (2000 mg/d). During the 9-mo trial in 40 patients with relapsing pouchitis, only 15% in the probiotic group relapsed while 100% in the placebo group relapsed[11]. A separate randomized trial of VSL#3® in patients with antibiotic-dependent pouchitis showed that 17 of 20 patients (85%) in the VSL#3® group maintained clinical remission, compared to remission in 1 of 16 patients (6%) in the placebo group[12]. However, in a recent post-market open-labeled trial of VSL#3® in 31 patients with antibiotic-dependent pouchitis, patients received 2 wk of treatment with ciprofloxacin followed by VSL#3®[71]. After 8 mo, 6 of the 31 patients (19%) were still taking VSL#3® and the remaining 25 patients (81%) stopped the agent mainly because of lack of efficacy or development of adverse effects[71].

Antibiotic-refractory pouchitis which is often difficult to treat, is a common cause of pouch failure. Since the patients typically do not respond to full-dose, single-agent antibiotic therapy, it is important to investigate contributing causes (in secondary pouchitis) related to failure to antibiotic therapy. Secondary causes of refractory disease include use of NSAID, concurrent *Clostridium difficile* or cytomegalovirus infection, celiac disease and other autoimmune disorders, cuffitis, CD of the pouch, pouch ischemia, and inflammatory polyps of the pouch[72]. There are no randomized trials in the literature for this category of pouchitis. For patients without obvious causes, treatment options include a prolonged course of combined antibiotic therapy, 5-aminosalicylates, corticosteroids, immunosuppressive agents or even biological therapy. Regimens reported in open-labeled trials include combined ciprofloxacin (1000 mg/d) with rifaximin (2000 mg/d)[73] or metronidazole (1000 mg/d)[74] or tinidazole (1000-1500 mg/d) for 4 wk[75]. However, maintenance of remission in this group of patients after the induction therapy with dual antibiotics remains a challenge[76]. Anti-inflammatory agents, immunomodulators, and biological therapy have been used to treat pouchitis. These agents include bismuth carbomer enemas, short-chain fatty acid enemas, and glutamine enemas, mesalamine enemas, oral budesonide[77], 6-mercaptopurine, and infliximab.

NATURAL HISTORY AND PROGNOSIS

The natural history of pouchitis is not entirely clear. In a study consisting of 100 consecutive UC patients who had restorative proctocolectomy with IPAA, 32 patients developed pouchitis, 5 had chronic refractory pouchitis, 2 of them had pouch failure after pouch resection[58]. Few studies were performed to identify the natural history of pouch and pouchitis. Patients with initial pouchitis almost uniformly respond to antibiotic therapy. However, relapse of pouchitis is common. Of the patients with acute pouchitis, 39% have a single acute episode that responds to antibiotic therapy whereas the remaining 61% of patients develop at least one recurrence[35]. Approximately 5% to 19% patients with acute pouchitis develop refractory or rapidly relapsing symptoms[78-80]. Here is a common scenario: the more frequent the episodes of pouchitis a patient has, the more often the antibiotic therapy is administered, the less likely the patient maintains favorable response to the treatment. The course of antibiotic-responsive pouchitis could evolve into antibiotic-dependent pouchitis followed by antibiotic-refractory pouchitis. Chronic refractory pouchitis is one of the most common causes for pouch failure. Although PSC is a risk factor for pouchitis[3,44,45], liver transplantation with post-transplant use of immunosuppressive agents does not appear to have adverse effects on the course of pouchitis[81,82]. In addition, chronic inflammation of the pouch and cuff may pose an increased risk of developing dysplasia or cancer[83,84].

In summary, pouchitis is the most common long-term adverse sequela of IPAA after restorative proctocolectomy. The natural history of pouchitis is yet to be defined. Patients with pouchitis can have a wide range of clinical presentations, disease courses, and prognoses. Accurate diagnosis and classification of pouchitis are the keys to appropriate management. Treatment of pouchitis is largely antibiotic-based. Maintenance of remission in antibiotic-dependent pouchitis and management of antibiotic-refractory pouchitis are a challenge. Secondary causes for refractory pouchitis should be excluded.

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BASIC RESEARCH

Effect of *Scutellariae Radix* extract on experimental dextran-sulfate sodium-induced colitis in rats

Ho-Lam Chung, Grace Gar-Lee Yue, Ka-Fai To, Ya-Lun Su, Yu Huang, Wing-Hung Ko

Ho-Lam Chung, Department of Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China
Grace Gar-Lee Yue, Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China
Ka-Fai To, Department of Anatomical and Cellular Pathology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China
Ya-Lun Su, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China
Yu Huang, Wing-Hung Ko, Department of Physiology and Li Ka Shing Institute of Health Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China
Supported by a direct grant for research from The Chinese University of Hong Kong, No. 2041075 awarded to Wing-Hung Ko
Correspondence to: Wing-Hung Ko, Department of Physiology and Li Ka Shing Institute of Health Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China. whko@cuhk.edu.hk
Telephone: +852-26096781 Fax: +852-26035022
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Abstract

AIM: To investigate the effect of *Scutellariae Radix* extract (SRE) on ulcerative colitis (UC) in rats induced by dextran-sulfate sodium (DSS).

METHODS: Colitis was induced in male Sprague-Dawley (SD) rats (170-180 g) by 4% dextran sulfate sodium (DSS, wt/v; MW 54000) in drinking water for 8 d. The treated rats received 4% DSS and SRE orally (100 mg/kg per day). Control rats received either tap water or SRE only. Macroscopic assessment which included body weight changes, fecal occult blood and stool consistency were determined daily. At the appointed time, the rats were sacrificed and the entire colons were removed. The colon length and the myeloperoxidase (MPO) activity were measured. The severity of colitis was graded by morphological and histological assessments. The ion transport activity of the colonic mucosa was assessed by electrophysiological technique.

RESULTS: Rats treated with oral administration of 4% DSS regularly developed clinical and macroscopic signs of colitis. Treatment with SRE relieved the symptoms, including the reduction in body weight, shortening and ulceration of the colon. Administration of SRE also significantly reduced the histological damage induced by DSS. Moreover, the *Isc* responses of the colonic mucosa to forskolin were suppressed after the induction of colitis. The stimulated ion transport activity of DSS-rats treated with SRE displayed significant improvement in the secretory responsiveness.

CONCLUSION: SRE was effective in treating acute DSS-induced ulcerative colitis, as gauged by reduced clinical disease, improved macroscopic and histological damage scores, and enhanced recovery of normal colonic secretory function.

Key words: Ulcerative colitis; *Scutellariae Radix*; Inflammatory bowel disease; Colonic ion transport; Traditional Chinese medicine

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INTRODUCTION

Ulcerative colitis is a worldwide, chronic, idiopathic, inflammatory bowel disease (IBD) of the rectal and colonic mucosa. In the past, this disease was thought to occur infrequently in the Asia Pacific region. However, new evidence is showing that IBD is on the rise in the region, including in Hong Kong and mainland China[1-3]. Although glucocorticoid and salicylazosulfapyridine have been mainly used for the treatment of this disease, their side effects remain a major clinical problem. Therefore, there is an increasing interest in using traditional Chinese medicines (TCM) as alternative therapy in addition to the conventional therapies that are used to treat UC[4].

The dried root of *Scutellaria baicalensis* Georgi (*Scutellaria Radix*, common name Huangqin) is widely used in TCM. It is officially listed in the Chinese Pharmacopoeia and is one of the most widely used Chinese herbal medicines for the treatment of bacterial infection of the respiratory and gastrointestinal tract. In Japan and China, *Scutellariae Radix* has been employed for centuries as an important medicine to treat chronic inflammatory and ulcerative disease. The main components of *Scutellariae Radix* (and of all *Scutellaria* species) are baicalein, baicalin and wogonin.

Scutellariae Radix and its major flavonoids possess multiple biological and pharmacological effects, including anti-inflammation[5], anti-viral[6], anti-tumor[7], anti-proliferative[8] and anti-bacterial[9], *etc*. Recent studies also suggest that *Scutellariae Radix*-containing TCM formula, such as Orengedoku-to (Huang Lian Jie Du Tang) may have therapeutic potential against murine colitis[10-12].

In this study, an experimental model of UC was established in SD rats using DSS. The effect of the total extract of *Scutellariae Radix* on DSS-rats was evaluated using macroscopic, histological, biochemical and electrophysiological assessments.

MATERIALS AND METHODS

Materials

Male SD rats, initially weighing 170-180 g, were housed five per cage and maintained in an animal holding room controlled at a constant temperature of 24℃ ± 2℃ with a relative humidity of 70% ± 5% and a 12 h light-dark cycle. Animals were fed on a standard pellet chow with free access to fresh tap water. The study was approved by the Animal Research Ethics Committee of our university. DSS was obtained from MP Biochemicals Inc. Hexadecyltrimethylammonium bromide, forskolin were obtained from Sigma. SRE was purified from the ground roots of *S. baicalensis* Georgi with hexane, acetone, and finally, with methanol as described previously[13,14].

Experimental design

The induction of colitis was modified from a previously described work[15]. The experiment lasted for 8 d. The rats were randomly divided into four groups. In the DSS Group, 4% DSS in drinking water *ad libitum* was given from d 0 to d 7. In DSS + SRE group, SRE (100 mg/kg per day) was orally administrated with exposure to 4% DSS drinking water. In the normal control group (Ctr), the rats had free access to a water bottle containing tap water. In Ctr + SRE group, SRE alone (100 mg/kg per day) was administered orally to the rats. In this model, the colonic damage was evaluated using macroscopic, histological, electrophysiological and biochemical assessments (see below). From d 3 to d 7, rats were sacrificed by CO_2 asphyxiation on each day. Postmortem, the entire colon was removed from the cecum to the anus and placed on an ice cold plate and cleaned of fat and mesentery. The length of each specimen was measured, which is an indirect marker of inflammation. The colon was divided into three parts (proximal, middle and distal) based on total length: 10% regions from three parts were fixed for histological examination; the distal portion was used for electrophysiological studies; and the adjacent distal 10% was snap-frozen in liquid nitrogen for later quantification of MPO activity.

Macroscopic assessment

Animals were checked daily for the three main clinical symptoms-body weight changes, stool consistency and fecal occult blood. Weight loss is usually observed in animals with colitis, thus body weight changes recorded could be an indicator for the severity of colitis. The colonic damage was quantified by a clinical scoring system assessing stool consistency and rectal bleeding[16]. For stool consistency, 0 points were given for well formed pellets, 2 points for pasty and semiformed stools that did not stick to the anus, and 4 points for liquid stools that did stick to the anus. Bleeding was scored 0 for no blood in hemoccult, 2 points for positive hemoccult, and 4 points for gross bleeding. These scores were added, forming a total clinical score that ranged from 0.0 (healthy) to 8.0 (maximal activity of colitis).

Histological assessment

For light microscopy, we used tissue samples from three parts (distal, middle and proximal) of colon of each animal fixed in 4% (40 g/L) buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 μm on a rotary microtome, mounted on clean glass slides and dried overnight at 37℃. The sections were cleaned, hydrated, and stained with hematoxylin and eosin (HE) for histological evaluation of inflammatory infiltrate and tissue damage, according to standard protocols, and the slides were coded to prevent observer bias during evaluation. All tissue sections were examined in a blinded fashion by two investigators. Histological damage was scored using the criteria of Siegmund B *et al*[17] which considers the inflammatory infiltrate (maximum score = 3) and tissue damage (maximum score = 3). Photographs of colon samples were digitized using a ZEISS Axioskop 2 plus camera. Analysis of the figures was carried out with Axio Vision 3.1 image analysis program.

Electrophysiological assessment

Mucosal ion transport was examined in colonic segments (approximately distal colon) mounted in Ussing chambers according to a well established protocol in our laboratory[18]. In brief, tissues (surface area = 0.45 cm^2) were bathed in 20 mL of warm (37℃), oxygenated Krebs buffer. The spontaneous potential differences were maintained at 0 mV by a voltage clamp amplifier (Physiologic Instruments), and the short-circuit current (I_{SC} in μAcm^{-2}) was continuously measured as an index of electrogenic ion transport. A transepithelial potential difference of 1 mV was applied periodically, and the resultant change in current was used to calculate the transepithelial resistance (R_t) using Ohm's law. Stimulated ion transport was evoked by the addition of an adenylate cyclase-activating agent, forskolin (1 μmol/L), to the mucosal bathing solution. In all instances, the effect of the treatment was recorded as the maximum change in I_{SC} (ΔI_{SC}) to occur within 5 min.

Biochemical assessment

The MPO activity was determined following a published protocol[19]. Briefly, frozen tissue samples were weighed and suspended in potassium phosphate buffer (20 mmol/L, pH 7.4) at a ratio of 50 mg tissue to 1 mL of buffer. Tissue was homogenized by a polytron tissue homogenizer three times for 20 s, and homogenate was decanted into sterile

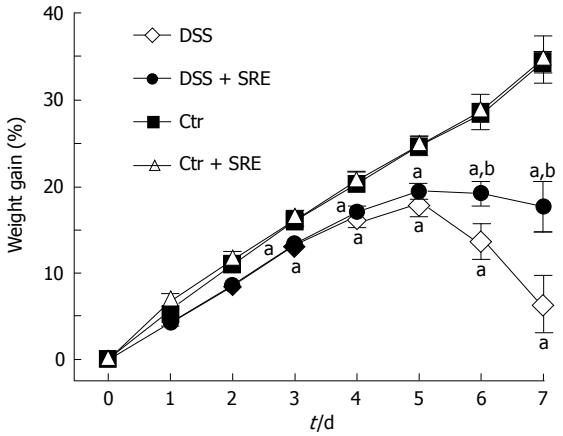

Figure 1 Figure showing the weight gain percentage (%) in different groups of rat from d 0 to d 7. Body weight was assessed daily and expressed as percentage increase of basal body weight. Values are expressed as the means ± SE, (n = 8), $^{a}P < 0.05$ *vs* Ctr group, $^{b}P < 0.05$ *vs* DSS group, one-way ANOVA followed by Tukey multiple comparison test.

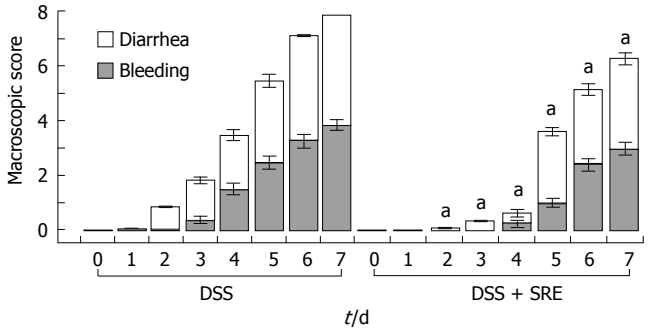

Figure 2 Ameliorative effect of SRE treatment on the time-course changes in the macroscopic score over the 8-d experimental period. The macroscopic score of Ctr and Ctr + SRE groups is 0 (data not shown). $^{a}P < 0.05$ *vs* DSS group. Non-parametric data are expressed as the means ± SE, Kruskal-Wallis One Way Analysis using Student-Newman-Keuls method (n = 6-8).

Eppendorf tubes and centrifuged at 10000 × g for 10 min at 4℃. The pellet was then resuspended in 1 mL potassium phosphate buffer (50 mmol/L, pH 6.0) containing 5 mg/mL hexadecyltrimethylammonium bromide (TMB) at a tissue concentration of 50 mg/mL. Samples were sonicated three times for 10 s, freeze-thawed three times, and centrifuged at 10000 × g for 5 min at 4℃. The reaction was started by mixing 20 μL of the supernatant at 25℃ with 30 μL TMB. After 110 s, the reaction was terminated by addition of 50 μL of 0.18 mol/L H_2SO_4. The change in absorbance was read at 450 nm. MPO activity (1 unit) was expressed as the amount of enzyme necessary for the degradation of 1 μmol H_2O_2/min per 100 mg tissue at 25℃.

Statistical analysis

All data are presented as means ± SE. One way ANOVA followed by post hoc statistics with Tukey test was used for statistical evaluation of the parametric data. Non-parametric data was analyzed by Kruskal-Wallis One Way Analysis using Student-Newman-Keuls method. $P < 0.05$ was considered as statistically significant. The values of n refer to the number of experiments undertaken using different rats. Non-parametric data are expressed as the means ± SE.

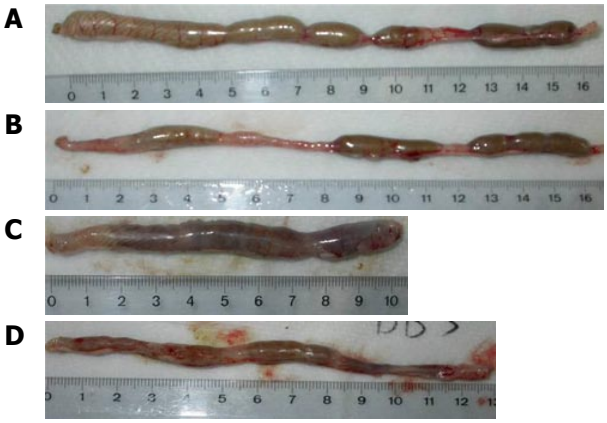

Figure 3 Macroscopic view of the colon showing the changes in colon length in different groups of rat on d 7. Not much difference was observed between the colon of the Ctr (**A**) and Ctr + SRE group (**B**), but significant difference could be found in the DSS group (**C**) which displayed extensive hyperemia and edema. In the DSS + SRE group, the shortening of colon was less severe (**D**) when compared with the control (**A**).

RESULTS

Macroscopic assessment

The weight gain % over the entire study period is shown in Figure 1. The weight gain % of rats in DSS group and DSS + SRE group were significantly lower than the Ctr group and Ctr + SRE group from d 3 until the end of experiment. The body weight of rats in DSS group then dramatically decreased from d 5 onwards. However, in the case of DSS + SRE group, the weight gain % became stabilized and the weight loss was found to be less severe than DSS group from d 6 to d 7. Figure 2 shows the macroscopic score recorded throughout the experimental period in DSS and DSS + SRE groups. Oral administration of SRE resulted in a significant reduction in the clinical activity of colitis compared with DSS-treated rats. Moreover, the occurrences of those clinical signs were found to be delayed in DSS + SRE group.

The colon length is a useful indication of colitis and is, therefore, measured as a marker of inflammation (Figure 3). After 8 d treatment with DSS in drinking water, there was a significant shortening of the colon length (Figure 3C) compared with the Ctr group (Figure 3A) and the Ctr + SRE group (Figure 3B). The oral administration of SRE significantly improved this inflammatory marker (Figure 3D). On d 7, the colon length of DSS group (11.2 cm ± 0.4 cm, n = 10) was significantly shorter than the control group (16.8 cm ± 0.6 cm, n = 8). The colon length of DSS-rats treated with SRE (13.5 cm ± 0.4 cm, n = 9), however, was significantly longer than that of untreated rats.

Histological assessment

Histological damage was evaluated by the grading method described above in Materials and Methods. The occurrence of UC was confirmed on the basis of histological damage and inflammatory infiltrate as shown in Figure 4. Figure 5 summarized the damage scores from DSS rats and DSS rats treated with SRE. The microscopic score of samples

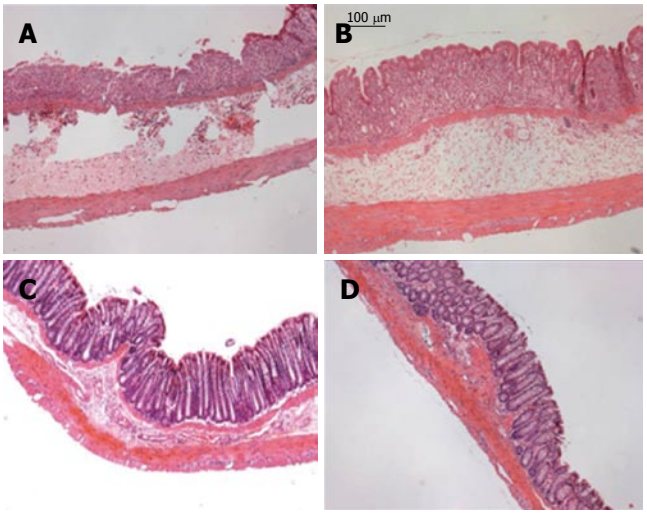

Figure 4 Histological sections from different groups of rats on d 7. **A**: DSS group showing extensive ulceration with a severe inflammatory cell infiltrate; **B**: DSS + SRE group showing recovery in the inflammatory cell infiltration with less severe ulceration; **C**: Non-colitic Ctr group showing the normal histology of the colon; **D**: Ctr + SRE group showing the normal morphology of the colon. (HE staining; original magnifications, x 100).

from different regions (distal, middle and proximal) of colon were not significantly different from each other (data not shown). The histological index began to increase on d 3 in both groups. However, the rats with SRE administration showed a significantly lower value than the untreated animals on d 6 and d 7.

Electrophysiological assessment

Electrogenic ion transport function was assessed by stimulating the colon with an adenylate cyclase activator, forskolin (1 μmol/L), using short-circuit current measurement technique (Figure 6). Under the experimental conditions, the increase in I_{SC} is mainly due to the cAMP-mediated Cl^- secretion *via* the cystic fibrosis transmembrane conductance regulator (CFTR)[18]. The basal I_{SC} and R_t in the tissues were recorded and these parameters were not significantly different (data not shown) when tissues from different groups were compared. On d 7, the secretory response to the cAMP-elevating agent was significantly diminished in tissues from rats treated with DSS (ΔI_{SC} = 4.94 ± 1.65 μAcm^{-2}, n = 6) when compared with Ctr (ΔI_{SC} = 60.97 ± 7.62 μAcm^{-2}, n = 8) and Ctr + SRE (ΔI_{SC} = 61.48 ± 6.78 μAcm^{-2}, n = 7). On the other hand, the reduced responsiveness was ameliorated in the colitic rats with SRE administration (ΔI_{SC} = 20.67 ± 3.30 μAcm^{-2}, n = 9).

Biochemical assessment

The MPO activity in the groups without DSS treatment remained at a low value throughout the entire experiment and there was no effect of SRE on the control (d 7: Ctr 0.09 ± 0.01 mU/mg, n = 6; Ctr + SRE 0.06 ± 0.0003 mU/mg, n = 5). In comparison, rats with colitis were accompanied by a significant increase in MPO activity (d 7: 0.52 ± 0.07 mU/mg, n = 8). On d 7, the increase in MPO activity (0.34 ± 0.05 mU/mg, n = 8) was significantly reduced in rats treated with SRE.

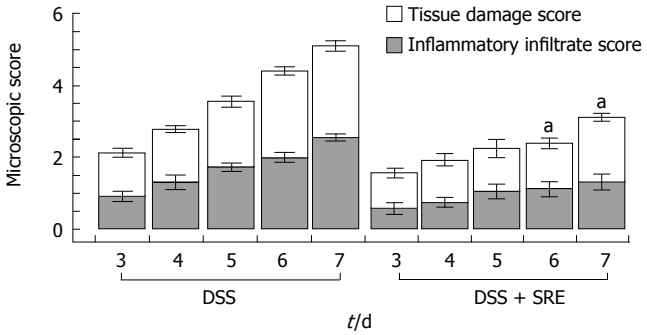

Figure 5 Ameliorative effect of SRE treatment on the time-course changes in the microscopic score from d 3 to d 7. The microscopic score of Ctr and Ctr + SRE groups is 0 (data not shown). $^aP < 0.05$ *vs* DSS group. Non-parametric data are expressed as the means ± SE, Kruskal-Wallis One Way Analysis using Student-Newman-Keuls method (n = 4-5).

DISCUSSION

Ulcerative colitis is an inflammatory disease that causes ulcerations of the mucosa in the colon. The incidence of UC is around 1 in 1000 people with a higher prevalence among Caucasians[20]. In the past, this disease has been considered to occur rarely in the Asia Pacific region, but recent evidence indicates that both UC and Crohn's disease (CD) are becoming increasingly prevalent in local populations[3]. For example, from 1991 to 2000, there has been a three-fold increase in the number of cases of UC in China[1]. In Hong Kong, from 1986-1989 to 1999-2001, there was also a three-fold increase in the incidence of CD in the Chinese population[2]. Although the etiology of IBD remains essentially unknown[21], results from many studies in human patients and animal models suggest that it may be related to an abnormal immune response in the gastrointestinal tract, possibly associated with genetic and environmental factors[22,23]. Although progress has been made in the overall management of UC, the pharmacological treatments that are available are still unsatisfactory. Glucocorticoids, sulfasalazine and immunosuppressive drugs have been mainly used for the treatment and maintenance of UC, but the side effects or toxicity of these drugs remain a major clinical problem[24]. As a result, there is an increasing interest in using TCM as alternative therapy in addition to the conventional therapies that are used to treat UC[25]. In Japan and China, the most commonly used alternative remedies are herbal and these have been widely used in patients with mild to moderate disease, as well as an adjunct to therapy in patients with moderate to severe disease[1]. Interestingly, a recent survey showed that one-third of both Chinese and Caucasian IBD patients had used complementary and alternative medicines and therapies[4]. Several TCM formulae have been shown to possess an anti-colitic effect in rats[26-28]. Recent studies suggest that *Scutellariae Radix* may have a pharmacological effect against murine colitis[10-12] and therefore in this study we aim to further evaluate the therapeutic effect of SRE on DSS-induced rat colitis.

In the present study, 4% DSS in drinking water was administrated for 8 d to induce acute colitis in rats. All DSS-treated rats showed numerous clinical symptoms such as body weight loss, diarrhea, bloody stools and shortening

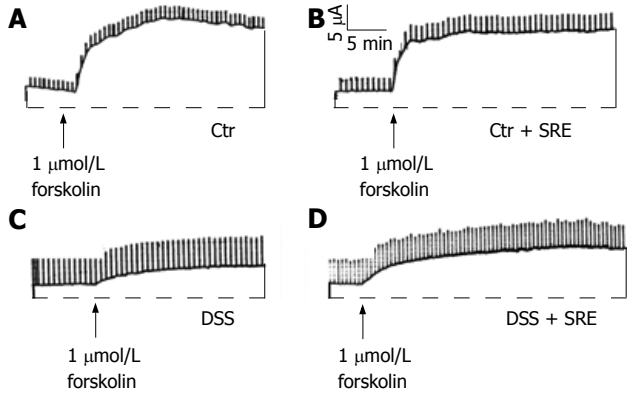

Figure 6 Representative tracings showing the change in I_{SC} evoked by forskolin in different groups of rat on d 7. All four groups showed an increase in ISC but with different magnitudes (**A-D**). The I_{SC} response was greatly reduced in the DSS group (**C**) when compared with the control (**A**). On the other hand, the reduction in secretory response appears to be ameliorated in the DSS + SRE group (**D**). The transient current pulses were the results of intermittently clamping the potential at 1 mV. The horizontal lines represent zero I_{SC}. The record is representative of at least six experiments.

of colon length. Body weight loss is a common symptom of UC because of the loss of body fluid and damage to the digestive system. In Ctr and Ctr + SRE groups, rats showed a steady increase in body weight throughout the experiment. In comparison, rats exposed to DSS had a lower rate of body weight increase followed by a dramatic decrease from d 5 onwards. Treatment with SRE to the colitic rats showed a less severe weight loss from d 6 to d 7. In DSS group, diarrhea and rectal bleeding occurred in d 1 and d 2, respectively. The SRE administration delayed the occurrence of both symptoms and with less severity. Colon shortening is always found in UC patients, which can act as an indirect marker of colonic inflammation. Although the colitic rats with SRE treatment also showed a decrease in colon length on d 7 when compared with the control, the shortening was much less severe than that of DSS rats. Taken together, the data suggested that SRE treatment could induce a decrease in the extent of colitis accompanied by reducing the severity and delaying the occurrence of the associated clinical symptoms.

Colitis induced by DSS was histologically characterized by severe disruption of tissue architecture, edema, a massive mixed immune cell infiltrate, ulceration and muscle thickening. To quantify the histological damage, we used a scoring system modified from a previous study[17]. We found that the overall histological damage of colitis was significantly reduced by the SRE treatment on d 6 and d 7 of the experiment. Together with the reduced MPO activity, our results showed that SRE treatment ameliorated the DSS-induced colitis possibly *via* its anti-inflammatory and protective effect against the colonic tissue damage.

The colonic epithelium, in addition to its absorptive and secretory properties, presents an efficient barrier to commensal flora and pathogens[29,30]. In fact, the secretion of water and electrolytes is one of the most important responses of the mucosa, purging the gut of offending agents and delivering anti-microbial mediators (e.g., antibodies) to the luminal surface[31]. In addition, mucus forms a gel layer covering the mucosal surface, and it has been hypothesized that changes in mucin structure and/or quantity may influence the protective functions of the mucosal surface, and affect the pathogenesis of IBD[32]. However, published data on the electrolyte transport mechanisms in an inflamed colon are inconsistent[33,34]. Some studies have documented the acute effects of immune and inflammatory agents in directly or indirectly stimulating intestinal anion secretion, while others do not support such a notion[35,36]. In most cases, however, disease models of colonic mucosa exhibit reduced ion transport responses to secretory agonists, especially in the setting of chronic inflammation[37-45].

The mechanism underlying the typical hyporesponsiveness of tissues from animal models of colitis has yet to be satisfactorily explained. For example, it has been reported that responsiveness to both Ca^{2+}- and cAMP-dependent secretagogues is reduced in mouse and rat colitis models when compared to normal tissue[37-45]. Similar reduction in secretory responsiveness, as measured by changes in I_{SC} has also been observed in tissue resections from patients with IBD[46,47]. It has been proposed that prolonged hyporesponsiveness to secretagogues is due to the upregulation of inducible nitric oxide synthase (iNOS), resulting in an ongoing synthesis of NO and chronic suppression of epithelial secretory function[41,42]. However, another recent study suggests that it may be related to a disturbance of the enteric nervous system resulting in defective mucosal cAMP production and inhibition of ionic secretion, although the epithelial secretory machinery (e.g., CFTR) appears to be normal[43]. Others suggested that prolonged hyporesponsiveness to secretagogues is due to the disruption of normal cholinergic control of ion secretion[40,48]. Nonetheless, intestinal secretion is an important component of mucosal defense. Reduced secretory responses will compromise the ability of the mucosal defense mechanism to clear bacteria, bacterial products, or antigens away from the epithelium, which may then predispose the colon to inflammation[49]. In this study, the I_{SC} responses of the colonic mucosa to forskolin were suppressed after the induction of colitis. Although the stimulated ion transport activity of DSS-rats treated with SRE was still reduced, they displayed improvement in the secretory responsiveness which may at least partly contribute to the therapeutic effect of SRE.

In summary, our findings indicated that SRE was effective in treating acute DSS-induced ulcerative colitis, as gauged by reduced clinical disease, improved macroscopic and histological damage scores, and enhanced recovery of normal colonic secretory function. The results support further evaluation of the therapeutic potential of SRE for the treatment of IBD.

ACKNOWLEDGMENTS

We thank Professor Woody WY Chan and Professor CH Cho for their technical advice in the performance of histological staining and MPO assay, respectively. We also thank Mr. Wallace CY Yip for his excellent technical help.

COMMENTS

Background

Scutellariae Radix, also known as Huangqin, is the dried root of *Scutellaria*

baicalensis Georgi (Lamiaceae). It is officially listed in the Chinese Pharmacopoeia and is one of the most widely used Chinese herbal medicines for the treatment of bacterial infection of the respiratory and gastrointestinal tract. In Japan and China, *Scutellariae Radix* has been employed for centuries as an important medicine. Although scientific evidence confirming the traditional use of *Scutellariae Radix* as an inflammatory modulator in experimental colitis is now accumulating, the physiological basis and the precise mechanism of action of *Scutellariae Radix* or its individual constituents remain largely unknown.

Research frontiers

Cytokine dysregulation is currently an important focus of both basic and clinical research in IBD, with recent immunologically based therapeutic interventions using highly specific agents demonstrating a promising clinical efficacy. The treatment of steroid-refractory Crohn's Disease with anti-TNF-α (infliximab) is an example of this kind of therapeutic approach. In addition, there is an increasing interest in using TCM as alternative therapy in addition to the conventional therapies that are used to treat IBD. However, there is still a paucity of scientific and clinical data so far to support the use of TCM in colitis patients. Therefore, there are unmet needs for further mechanistic, pharmacological and pharmacokinetic studies to delineate the biological basis underlying the therapeutic effects of TCM. More controlled clinical trials of the potential efficacy and safety of herbal TCM therapies are also required.

Innovations and breakthroughs

The authors showed for the first time that the therapeutic potential of *Scutellariae Radix* extract on experimental colitis may be related to the restoration of ion transport function of the colonic mucosa.

Applications

The results support further evaluation of the therapeutic potential of this herbal extract and its active component(s) for the treatment of IBD.

Terminology

Colonic ion transport: Intestinal fluid secretion is a passive process driven by osmotic forces generated by ion transport. In the colon, the main determinant of a luminally-directed osmotic gradient is the mucosal transport of chloride ions into the lumen. Intestinal secretion is an important component of mucosal defense. Reduced secretory responses will compromise the ability of the mucosal defense mechanism to clear bacteria, bacterial products, or antigens away from the epithelium, which may then predispose the colon to inflammation.

Peer review

This is an interesting paper which shows the anti-inflammatory effect of *Scutellariae Radix* on experimental colitis. It also demonstrates that TCM has a scientific and biological basis for its effectiveness.

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BASIC RESEARCH

Induction of apoptosis by artemisinin relieving the severity of inflammation in caerulein-induced acute pancreatitis

Ming Zhao, Dong-Bo Xue, Biao Zheng, Wei-Hui Zhang, Shang-Ha Pan, Bei Sun

Ming Zhao, Dong-Bo Xue, Biao Zheng, Wei-Hui Zhang, Bei Sun, Department of General Surgery, First Clinical College of Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Shang-Ha Pan, The Key Laboratory of Hepatic-spleenic Surgery of Heilongjiang Province, Harbin 150001, Heilongjiang Province, China
Supported by the Natural Science Foundation of Heilongjiang Province, China, No. D0227
Correspondence to: Wei-Hui Zhang, Department of General Surgery, First Clinical College, Harbin Medical University, Harbin 150001, Heilongjiang Province, China. zhangweihui626@hotmail.com
Telephone: +86-451-53658828 Fax: +86-451-53658828
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Abstract

AIM: To observe the apoptosis and oncosis of pancreatic acinar cells and secondary inflammatory reaction in pancreatic tissue from rats with acute pancreatitis (AP), and the influences of artemisinin on them.

METHODS: AP was induced by 4 intraperitoneal injections of caerulein at 1 h intervals. To induce apoptosis, solution of artemisinin (50 mg/kg) was given intraperitoneally 1, 12, 24 and 36 h after the last caerulein injection. Histological examination of impairment of pancreatic tissue and detection of serum amylase were performed to evaluate the severity of acute pancreatitis. Apoptosis and oncosis were detected with acridine orange (AO) and ethylene dibromide (EB) staining. Caspase-3 and myeloperoxidase (MPO) activity were measured by colorimetric assay. Nuclear factor-kappa B (NF-κB) activation was detected by flow cytometry. Macrophage inflammatory protein-1α (MIP-1α) protein was measured by Western blot. Interleukin-1β (IL-1β) mRNA was detected by RT-PCR.

RESULTS: Addition of artemisinin increased the number of apoptotic cells (11.7% ± 1.4% *vs* 6.3% ± 0.7%, $P < 0.05$), while reduced the number of oncotic cells (13.0% ± 2.4% *vs* 17.5% ± 2.2%, $P < 0.05$). The activity of caspase-3 speeded up (1.52 ± 0.21 *vs* 1.03 ± 0.08, $P < 0.05$), the pancreas pathological impairment was relieved (3.0 ± 0.5 *vs* 4.0 ± 0.5, $P < 0.05$) and the level of serum amylase decreased (5642 ± 721 U/dL *vs* 7821 ± 653 U/dL, $P < 0.05$). The activation of NF-κB (29% ± 4.1% *vs* 42% ± 5.8%), MIP-1α protein (3.7 ± 0.5 *vs* 5.8 ± 0.7), MPO (0.52 ± 0.06 U/g *vs* 0.68 ± 0.09 U/g), IL-1β mRNA (1.7 ± 0.3 *vs* 2.4 ± 0.4) in the apoptosis inducing group was obviously decreased ($P < 0.05$).

CONCLUSION: Inducing apoptosis can relieve pathological impairment and inflammatory reaction in AP rats.

Key words: Pancreatitis; Apoptosis; Inflammation mediators; Chemokines; Artemisinin

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INTRODUCTION

Many factors lead to acute pancreatitis (AP). A series of cascade reactions of inflammatory mediators and over-activation of leukocytes are the important causes for systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS)[1]. Although anti-cytokine therapy is able to relieve the severity of AP, it is difficult to block each pathway due to the complicated network of cytokines[2]. Therefore, knowing how to inhibit the initial inflammatory reaction is the key to the treatment of AP. It was reported that inflammatory reaction is correlated to the death modes of pancreatic acinar cells[3]. If death of pancreatic acinar cells occurs in the mode of apoptosis, the cell membrane is intact and there is no release of inflammatory mediators and pancreatin, the inflammatory reaction may be mild[4]. However, if death of pancreatic acinar cells occurs in the mode of oncosis, various pancreatin and inflammatory mediators may release, thus causing a variety of inflammatory cell aggregations and inducing intense inflammatory reactions[5]. If we can induce apoptosis and reduce oncosis, intense inflammatory reactions may be inhibited.

In this study, apoptosis of pancreatic acinar cells was induced by the apoptosis inductor-artemisinin. Changes in apoptosis, oncosis and secondary inflammatory reaction were observed.

MATERIALS AND METHODS

Experimental protocol

Twenty-four male Wistar rats (200 g ± 20 g) were provided by the Animal Research Center of the First Clinical College of Harbin Medical University (Harbin, China), and divided into 3 groups (8 rats in each group): control group, AP group, and artemisinin-treated group (apoptosis inducing group). AP was induced by 4 intraperitoneal injections of caerulein (20 μg/kg, Sigma, USA) at 1 h intervals. To induce apoptosis, solution of artemisinin (2 mg/kg, Huaxin, Sichuan, China) was given intraperitoneally 1, 12, 24 and 36 h after the last caerulein injection. The control rats were only given saline solution. Forty-eight hours after the final injection of caerulein, rats were anaesthetized with sodium pentobarbital (40 mg/kg), and then a laparotomy was performed with the pancreas rapidly removed for further analyses.

Histological examination of pancreatic tissue

Samples of pancreatic tissue were fixed in 20% formaldehyde and processed for paraffin histology. After staining with hematoxylin and eosin (HE), histological grading of interlobular edema, inflammatory infiltration, parenchyma hemorrhage, parenchyma necrosis and vacuolization was valued as previously described[6], then a pathologic score was calculated based on these light microscopic examinations.

Measurement of serum amylase

Serum was collected from the rats for amylase measurement. Amylase level was determined using a commercial chromatometric kit (Jiancheng, Nanjing, China).

Detection of apoptosis and oncosis with acridine orange (AO) and ethylene dibromide (EB) staining

Pancreatic acinar cells were isolated from Wistar rats by two-step collagenase digestion[7] and loaded onto slides with AO (10 μg/mL, Sigma, USA) and EB (10 μg/mL, Sigma, USA) for 10 min. The slides were scanned under confocal laser microscope (Zeiss, Germany) and 500 cells were counted under fluorescent microscope (Nikon, Japan).

Measurement of caspase-3 activity by colorimetric assay

Caspase-3 activity was measured using a colorimetric assay kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions. After isolation by two-step collagenase digestion, pancreatic acinar cells were mixed with 50 μL lysis buffer, the supernatant was mixed with 5 μL caspase substrate and 50 μL reaction buffer, and incubated at 37℃ for 4 h in the dark. Fluorescence intensity of the caspase substrate was measured photometrically at 405 nm.

Detection of NF-κB by flow cytometry

Fresh pancreatic tissues were sheared into pieces of 1.0 mm^3 with scissors, and centrifuged at 200 × *g* for 5 min. Then 10 mL detergent solution containing 1% TritonX-100 (Sigma,USA) was added, and stored in a refrigerator at 4℃ for 18 h, then filtered through 50 μm nylon meshes. For fluorescent labeling, 1 μL RNAase was added into 50 μL nucleus suspension and water-bath at 37℃ for 30 min, then 40 μL NF-κB p65 monoclonal antibody (Santa Cruz, USA) was added and incubated at room temperature for 20 min. The samples were treated with 1 μL FITC-labeled second antibody (Jackson Immuno Research, USA) and incubated at room temperature for another 20 min. After treated with 20 μL propidium iodide (PI, Sigma, USA) for 30 min in a dark room, the samples were analyzed using a FACScan flow cytometer (Becton Dicknson, USA).

Measurement of macrophage inflammatory protein-1α (MIP-1α) by Western blot

MIP-1α was detected by Western blot analysis, total protein extract was separated by 10% SDS/PAGE before it was transferred electrophoretically (100 V, 1 h) to hybond C membrane. The membranes were probed with 10 μL anti-MIP-1α monoclonal antibody (Santa Cruz, USA) in TBS-T (1:500), and the immunocomplexed membranes were re-probed at room temperature for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Jackson Immuno Research, USA) in TBS-T (1:500), with 5% blocking reagent. At last, the immunoreactive proteins were visualized using the ECL Western blot analysis system (Amersham, UK).

Chromatometric detection of myeloperoxidase (MPO) activity

The pancreatic tissue was frozen in liquid nitrogen and homogenated. MPO activity was detected with a chromatometric kit (Jianchen, Nanjing, China) following the manufacturer's instructions, and data were expressed as the change in absorbance at 460 nm.

Detection of interleukin-1β (IL-1β) mRNA by semi-quantitive RT-PCR

Total RNA was extracted from pancreatic tissue using Trizol reagent (Invitrogen, USA) and reversely transcriped into cDNA according to the instructions of the kit (Promega, USA). The resulting cDNA was used as a template for subsequent polymerase chain reaction (PCR). The sequences of rat-specific primers for IL-1β (519 bp) are as follows: 5'CCAGGATGAGGACCCAAGCA3' (sense), 5'TCCCGACCATTGCTGTTTCC3' (antisense). Housekeeping gene β-actin (348 bp) was used as a controller, 5'CATCACCATTGGCAATGAGCG3' (sense), 5'CTAGAAGCATTTGCGGTCGGAC3' (antisense). The PCR products were resolved in 1.0% agarose gel for electrophoresis and photographed under ultraviolet transillumination and the intensity of PCR products was measured using a video image analysis system.

Statistical analysis

Data were expressed as mean ± SD. Differences between the groups were tested for significance by one-way analysis of variance (ANOVA), and intergroup comparison was made by Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

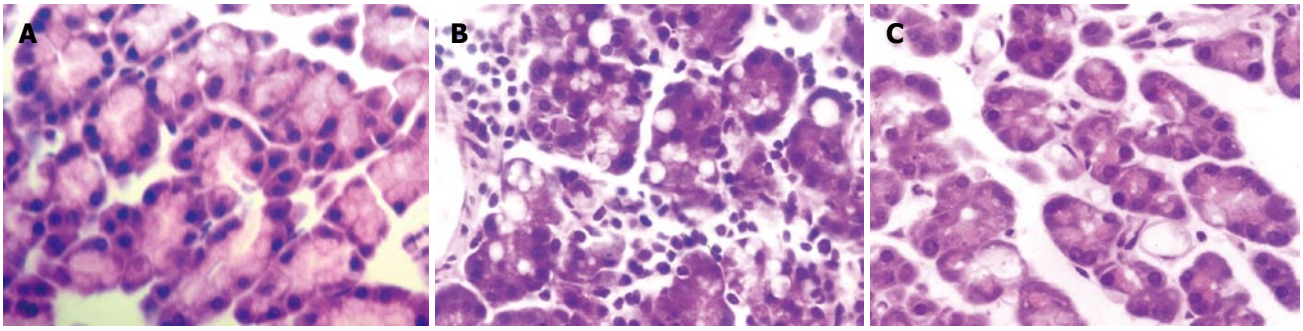

Figure 1 Representative HE-stained pancreatic tissue from the rats (original magnification × 40) in the control group (**A**), AP group (**B**) and artemisinin-treated group (**C**).

Table 1 Detection of cell death pathway and pathologic changes in different groups (mean ± SD, n = 8)

	Pathological score	Amylase (U/dL)	Apoptosis index (%)	Oncosis index (%)	Caspase-3 activity
Control	0.25 ± 0.03	2887 ± 298	1.5 ± 0.3	2.1 ± 0.3	0.35 ± 0.04
AP	4.0 ± 0.5[a]	7821 ± 653[a]	6.3 ± 0.7[a]	1.03 ± 0.08[a]	17.5 ± 2.2[a]
Artemisinin	3.0 ± 0.5[c]	5642 ± 721[c]	11.7 ± 1.4[c]	13.0 ± 2.4[c]	1.52 ± 0.21[c]

AP: Acute pancreatitis. [a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* AP group.

RESULTS

Histological changes after induction of apoptosis

Pancreatic tissue was normal in the control group with a low pathological score. In the AP group, pancreatic tissue displayed lobular mesenchymal rarefaction, edema and inflammatory cell infiltration. In contrast, in the artemisinin-treated group, edema and inflammatory cell infiltration were significantly relieved compared with the AP group (Figure 1). The pathological score showed alleviated pathological impairment in pancreatic tissue after treated with artemisinin ($P < 0.05$ *vs* the AP group) (Table 1).

Effect of artemisinin on induction of apoptosis and avoidance of oncosis

As shown in Figure 2, the nuclei of normal cells showed normal morphology of green fluorescence, while apoptotic cells showed shrunk, condensed or splitted nuclei (green). EB could be resisted by the intact cytoplasmic membrane of normal and apoptotic cells. EB could penetrate the cytoplasmic membrane of oncotic cells, and stain the nuclei of orange-stained cells. So, the apoptotic index or oncotic index, i.e., the number of apoptotic cells or oncotic cells per 100 cells, could be calculated (Figure 3). The results indicate that only sporadic apoptotic or oncotic cells were observed in the control group, but more in the AP and artemisinin-treated groups. In the AP group, there were less apoptotic cells and more oncotic cells. The number of apoptotic cells increased and the number of oncotic cells decreased significantly in the artemisinin-treated group ($P < 0.05$) (Table 1).

Effect of artemisinin on caspase-3 activity

The activity of caspase-3 in isolated pancreatic acinar cells was low in the control group, and high in the AP group, which was significantly elevated after apoptosis was induced by artemisinin ($P < 0.05$) (Table 1).

Influence of apoptosis on NF-κB activation

Activation of NF-κB in normal pancreatic nuclei was significantly higher in the AP group than in the control group ($P < 0.05$) (Figure 4, Table 2).

Influence of apoptosis on MIP-1α

MIP-1α level was low in the control group and high in the AP group. It was obviously lower in the artemisinin-treated group than in the AP group ($P < 0.05$) (Figure 5, Table 2).

Influence of apoptosis on IL-1β mRNA expression

The expression of IL-1β mRNA was low in the control group and high in the AP group. It was lower in the artemisinin-treated group than in the AP group ($P < 0.05$) (Figure 5, Table 2).

Changes in MPO contents after induction of apoptosis

Since each neutrophil granulocyte contains a certain quantity of MPO, detection of MPO in pancreatic tissue reflects the degree of neutrophil infiltration of the tissue. In our study, the MPO concentration was low in the control group and high in the AP group. However, it was down-regulated after induction of apoptosis ($P < 0.05$) (Table 2).

DISCUSSION

AP characterized not only by pancreas impairment, but also by inflammatory cell infiltration and release of various kinds of inflammatory mediators, can develop into SIRS and MODS in some cases and endanger their life[8,9]. Initiation of inflammatory reaction is related to the death pathway of impaired pancreatic acinar cells[10]. Since the conception of apoptosis was proposed by Kerr in 1972[11], apoptosis has been extensively studied. In recent years, attention has been paid to another cell death pathway-oncosis, and it was gradually realized that oncosis makes no less sense than apoptosis[12]. Oncosis has a feature of cell swelling, and cell membrane integrity is destroyed and DNA is split into non-specific fragments. Finally the cells are dissolved accompanying inflammatory reaction. In some physiological and pathological processes, these two kinds of death pathways exist simultaneously, and may

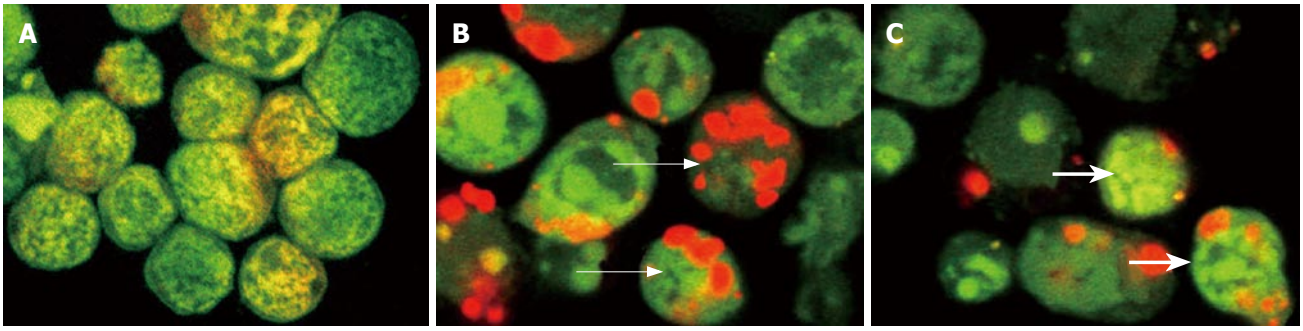

Figure 2 Isolated pancreatic acinar cells stained with AO and EB, and scanned under confocal laser microscope (original magnification × 100) in the control group (**A**), AP group (**B**), and artemisinin-treated group (**C**). Fine arrows indicate oncotic cells, and thick arrows indicate apoptotic cells.

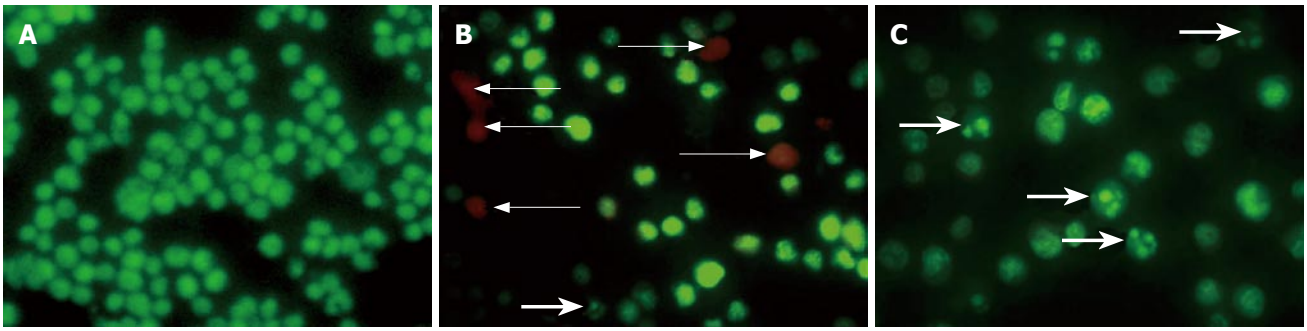

Figure 3 Isolated pancreatic acinar cells stained with AO and EB, and observed under fluorescent microscope (original magnification × 40) in the control group (**A**), AP group (**B**), and artemisinin-treated group (**C**). Fine arrows indicate oncotic cells, and thick arrows indicate apoptotic cells.

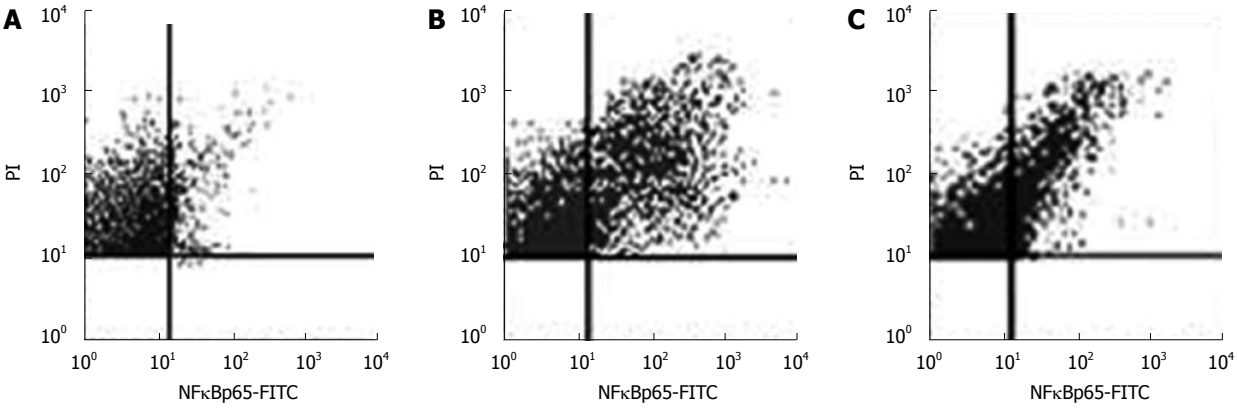

Figure 4 Flow cytometric analysis of NF-κB activation in the control group (**A**), AP group (**B**), and artemisinin-treated group (**C**).

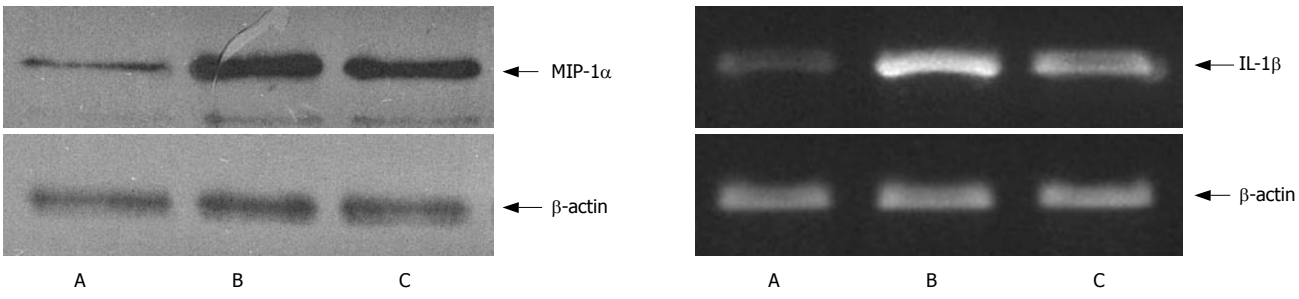

Figure 5 MIP-1α measured by Western blot and IL-1β mRNA detected by RT-PCR in the control group (A), AP group (B), and artemisinin -treated group (C).

Table 2 Inflammatory response of pancreatic tissue in different groups (mean ± SD, n = 8)

	NF-κB activation (%)	MIP-1α protein	MPO (U/g)	IL-1β mRNA
Control	4.7 ± 0.6	0.9 ± 0.1	0.19 ± 0.03	0.5 ± 0.1
AP	42 ± 5.8[a]	5.8 ± 0.7[a]	0.68 ± 0.09[a]	2.4 ± 0.4[a]
Artemisinin	29 ± 4.1[c]	3.7 ± 0.5[c]	0.52 ± 0.06[c]	1.7 ± 0.3[c]

MIP: macrophage inflammatory protein; MPO: myeloperoxidase. [a]P < 0.05 *vs* control group; [c]P < 0.05 *vs* AP group.

interconvert to each other under certain conditions[13]. Oncotic cells may release much more entocytes (including digestive enzyme and inflammatory active medium). It not only destroys the local tissue, but also activates mononuclear cells, leading to SIRS. However, apoptosis may not cause secondary inflammation. During AP, the patient has his or her own self-regulating mechanism. Under certain conditions, the apoptotic signal conduction pathway can be initiated, and more apoptosis will be induced and harmful effects of oncosis may be relieved. However, if AP develops quickly or the patients' self-regulation is in disorder, the apoptosis signal conduction pathway cannot be initiated in time, causing predominant oncosis, leading to aggravation of the disease. This has been proved by Kaiser *et al*[14], who found that when apoptosis is inhibited by cycloheximide, AP obviously aggravates. However, Bhatia *et al*[15] induced apoptosis during their experiment, resulting in the relief of AP. It was reported that one of the therapeutic mechanisms of somatostatin analogue, the most effective drug for AP, is to induce apoptosis of impaired pancreatic acinar cells[16]. Whether AP can be controlled by inducing apoptosis of pancreatic acinar cells has been extensively studied.

Artemisinin is an important active component of traditional Chinese medicine which can induce apoptosis[17,18]. Hahm *et al*[19] found that apoptotic index is elevated but pathological changes in AP rats treated with DA-9610 (an extract from Artemisia Asiatica). In this study, artemisinin was used as an apoptosis inductor. After artemisinin was added into pancreatic acinar cells stimulated by caerulein, apoptosis and oncosis were detected with AO and EB staining, the number of apoptotic cells increased, but the number of oncotic cells decreased. Caspase-3 is a key molecule in the process of apoptosis. Generally, the precursor of caspase-3 in cells is incompetent. However, if it is activated, it can cleave important structural and functional proteins inside the cells and cause chromosome condensation, DNA fragmentation, nuclear membrane rupture, *etc*, finally resulting in apoptosis. The presence of activated caspase-3 indicates that apoptosis is at the irreversible stage[20]. Nam *et al*[21] found that artemisinin can induce apoptosis by up-regulating caspase-3. Our study also showed that the activity of caspase-3 in pancreatic acinar cells was obviously elevated after artemisinin was added, indicating that artemisinin may promote apoptosis. We examined the pancreatic tissue with HE staining and found that after induction of apoptosis, infiltration of inflammatory cells decreased and the pancreas impairment was relieved. Based on this understanding, we observed the degree of inflammatory reaction after induction of apoptosis.

We detected transcription factor-NF-κB which regulates synthesis of many inflammatory mediators and cytokines[22]. NF-κB is a protein that regulates gene transcription, participates in regulating many inflammatory factors, and evokes immune and inflammatory reactions[23]. NF-κB plays a key role in the development of AP[24]. In our study, activation of NF-κB in the apoptosis inducing group was obviously decreased, compared with the AP group, indicating that activation of NF-κB can be decreased by inducing apoptosis and reducing oncosis. The conserved sequence of MIP-1α combined with NF-κB exists in its promoter region[25], suggests that MIP-1α may be one of the downstream targets regulated by NF-κB. MIP-1α was detected by Western blot assay in this study, proving that if apoptosis is induced, MIP-1α can be inhibited. MIP-1α is a CC-type chemotatic factor and plays an important role in recruiting mononuclear cells and lymphocytes[26]. Just as the "over-activation of leucocyte theory" proposed by Rindernech *et al*[27], AP aggravates because inflammatory cells are over-activated, and these activated cells such as granulocytes and macrophages, play a great role in the development of AP. Therefore, MPO (the marker of neutrophils) and IL-1β (the inflammatory factors generated mainly by mononuclear macrophages)[28] were detected in this study, indicating that MPO is obviously decreased in pancreas tissue after induction of apoptosis, reducing neutrophil recruition and infiltration to pancreas tissue. IL-1β is a kind of proinflammatory cytokines mainly generated by macrophages in pancreas when AP occurs. IL-1β can activate neutrophils, up-regulate the expression of surface adhesion molecules of lymphocyte and endotheliocytes[29]. Fink *et al*[30] reported that release of pancreatic amylase and necrosis of pancreas tissue are obviously decreased by blocking IL-1 receptor, demonstrating that IL-1 is essential to inflammation and development of AP. Our study proved that after induction of apoptosis, the level of IL-1β mRNA in pancreas tissue was low, indicating that infiltration and activation of macrophages are decreased and inflammatory reaction is inhibited after induction of apoptosis.

In conclusion, infiltration of inflammatory cells and generation of inflammatory cytokines can be decreased by inducing apoptosis and reducing oncosis of pancreatic acinar cells.

COMMENTS

Background

One of the greatest findings in AP is the initiation of cytokine network in AP patients that promotes occurrence of SIRS and MODS. Efforts have been made to alleviate pathological changes in AP by inhibiting the cytokine network. Since cytokine network is so complex that it is impossible to block all the pathways, it may pave a new way for the treatment of AP to obstruct the cytokine chain reactions. It was reported that the cytokine network can be obstructed by inducing apoptosis, decreasing oncosis and release of endocellular enzyme, suggesting that any drugs regulating apoptosis may be used in the treatment of AP.

Research frontiers

It has been verified that AP worsens when apoptosis of pancreatic acinar cells is

inhibited by cycloheximide, and that AP is alleviated when apoptosis is induced. It was reported that the severity of AP is associated with the degree of oncosis. Hahm *et al* found that there is an elevated apoptotic index but extenuated pathological changes in AP rats after treated with DA-9610 (an extract from Artemisia Asiatica), suggesting that artemisinin can regulate cell death and can be used in the treatment of AP.

Innovations and breakthroughs

When the concept of apoptosis was first put forward by Kerr in 1972, a lot of studies have been performed with it. In recent years, more and more attention has been paid to oncosis, showing that the importance of oncosis is no less than that of apoptosis. In traditional Chinese medicine, some important prescriptions have been successfully applied in AP treatment, and one of the mechanisms is to suppress inflammatory response and induce apoptosis. Artemisinin is an important active component of traditional Chinese medicine which can induce apoptosis. In this study, we observed the regulatory effect of artemisinin on apoptosis and oncosis of pancreatic acinar cells and its therapeutic effect on AP.

Applications

Artemisinin can induce apoptosis and reduce oncosis of pancreatic acinar cells at the onset of AP, thus repressing the intense inflammatory reactions, such as SIRS and MODS. Therefore, there is a bright prospect for artemisinin in the treatment of AP.

Terminology

Oncosis, or cellular swelling, is a pathological process of cell death, in which the completeness of cell membrane is destructed and DNA is split to non-specific fragments, ultimately leading to cell lysis complicated by inflammatory reactions.

Peer review

The effect of artemisinin on acute pancreatitis was analyzed. The data presented are interesting.

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CLINICAL RESEARCH

Rational prescription of drugs within similar therapeutic or structural class for gastrointestinal disease treatment: Drug metabolism and its related interactions

Quan Zhou, Xiao-Feng Yan, Zhong-Miao Zhang, Wen-Sheng Pan, Su Zeng

Quan Zhou, Xiao-Feng Yan, Zhong-Miao Zhang, Department of Clinical Pharmacology & Clinical Pharmacy, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China
Wen-Sheng Pan, Department of Gastroenterology, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China
Su Zeng, Department of Pharmaceutical Analysis & Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, Zhejiang Province, China
Supported by the Scientific Research Project of Zhejiang Provincial Bureau of Education, No. 20061449, No. 20010535
Correspondence to: Quan Zhou, Associate Professor, Department of Clinical Pharmacology & Clinical Pharmacy, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China. zhouquan142602@zju.edu.cn
Telephone: +86-571-87783891 Fax: +86-571-87213864
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Abstract

AIM: To review and summarize drug metabolism and its related interactions in prescribing drugs within the similar therapeutic or structural class for gastrointestinal disease treatment so as to promote rational use of medicines in clinical practice.

METHODS: Relevant literature was identified by performing MEDLINE/Pubmed searches covering the period from 1988 to 2006.

RESULTS: Seven classes of drugs were chosen, including gastric proton pump inhibitors, histamine H_2-receptor antagonists, benzamide-type gastroprokinetic agents, selective 5-HT_3 receptor antagonists, fluoroquinolones, macrolide antibiotics and azole antifungals. They showed significant differences in metabolic profile (i.e., the fraction of drug metabolized by cytochrome P450 (CYP), CYP reaction phenotype, impact of CYP genotype on interindividual pharmacokinetics variability and CYP-mediated drug-drug interaction potential). Many events of severe adverse drug reactions and treatment failures were closely related to the ignorance of the above issues.

CONCLUSION: Clinicians should acquaint themselves with what kind of drug has less interpatient variability in clearance and whether to perform CYP genotyping prior to initiation of therapy. The relevant CYP knowledge helps clinicians to enhance the management of patients with gastrointestinal disease who may require treatment with polytherapeutic regimens.

Key words: Cytochrome P450; Pharmacokinetics; Drug metabolism; Genotype; Polymorphism; Drug interaction; Pharmacotherapy; Gastrointestinal diseases

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INTRODUCTION

More and more drugs within the similar therapeutic or structural class are emerging and it is essential to compare the alternative drug choices according to their efficacy, safety, suitability and cost. However, irrational prescription is common in many countries. Drug metabolism and its related interactions are most prone to be ignored in clinical practice. Actually, metabolism by cytochrome P450 (CYP) represents an important clearance mechanism for the majority of drugs, thus affecting their oral bioavailability, duration and intensity of pharmacological action[1]. The metabolic profile of a drug depicts its amount metabolized by CYP, CYP reaction phenotype, impact of CYP genotype on interindividual pharmacokinetics (PK) variability and CYP-mediated drug-drug interaction potential. It is closely related to the three-dimensional chemical structure of drug and may exhibit significant differences among drugs within the similar therapeutic or structural class, although the efficacy of these similar drugs do not show sharp differences at the dose used clinically[2,3]. The voluntary market withdrawal of cerivastatin by Bayer and withdrawal of medications such as terfenadine, astemizole, cisapride, and mibefradil from the market by the Food and Drug Administration (FDA) further demonstrate the relevance of metabolic drug-drug interaction profile. Although the FDA has published guidance for *in vitro* and *in vivo* drug metabolism/drug interaction

studies in the drug development process[4,5], systematic summary is not yet available on metabolic differences in market products within the similar therapeutic or structural class. This review focuses on seven classes of drugs for gastrointestinal diseases treatment and aims to help clinicians realize what kind of drug has less interpatient variability in clearance, whether to perform CYP genotyping prior to the initiation of therapy, and how to enhance the management of patients on polytherapy regimens.

MATERIALS AND METHODS

Seven classes of drugs for gastrointestinal diseases treatment were chosen, including gastric proton pump inhibitors, histamine H_2-receptor antagonists, benzamide-type gastroprokinetic agents, selective 5-HT_3 receptor antagonists, fluoroquinolones, macrolide antibiotics and azole antifungals. Relevant literature, focusing on drug metabolism, metabolic interaction potentials and clinical events of adverse drug reactions and treatment failures caused by drug-drug interaction, was identified by performing MEDLINE/Pubmed searches covering the period from 1988 to 2006.

RESULTS

Gastric proton pump inhibitors

Proton pump inhibitors (or "PPI"s) are a group of drugs widely prescribed for the treatment of acid-related diseases such as peptic ulcer, gastroesophageal reflux disease (GERD), nonsteroidal anti-inflammatory drug induced gastropathy and Zollinger-Ellison syndrome. Currently used PPIs in clinical practice are as follows: omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole. All are benzimidazole derivatives (Figure 1). Schematic depiction of metabolic differences among four PPIs is described in Figure 2.

Lansoprazole, omeprazole and pantoprazole are all primarily metabolized by CYP2C19, an isoenzyme that exhibits genetic polymorphism with 15%-20% of Asian populations being poor/slow metabolizers, whereas the prevalence is much lower (3%-5%) among Caucasians and Blacks[6]. Their PK behaviors are all dependent on CYP2C19 genotype. $AUC_{po(PM)}/AUC_{po(EM)}$, the ratio of parent drug area-under-the concentration *vs* time curve after oral dosing (AUC_{po}) derived from poor metabolizers (PM) and extensive metabolizers (EM), is 7.4, 3.7 and 6.0 for omeprazole, lansoprazole and pantoprazole, respectively[7]. CYP2C19 polymorphism is also a major predictor of treatment failures in patients receiving lansoprazole-, omeprazole- or pantoprazole based polytherapy for eradication of *H pylori*[8].

Omeprazole has also been known as a potent inhibitor of CYP2C19, and may cause pharmacokinetic interactions with other CYP2C19 substrates such as diazepam, phenytoin and moclobemide[9-11]. Both lansoprazole and omeprazole also induce CYP1A2 *in vitro*[12]. Omeprazole can reduce clozapine plasma concentrations by 40%[13]. However, concomitant intake of omeprazole or lansoprazole at high therapeutic doses does not affect the PK behavior of theophylline and caffeine[14,15]. The underlying explanation of the discrepancy may be that inducibility of CYP1A2 by

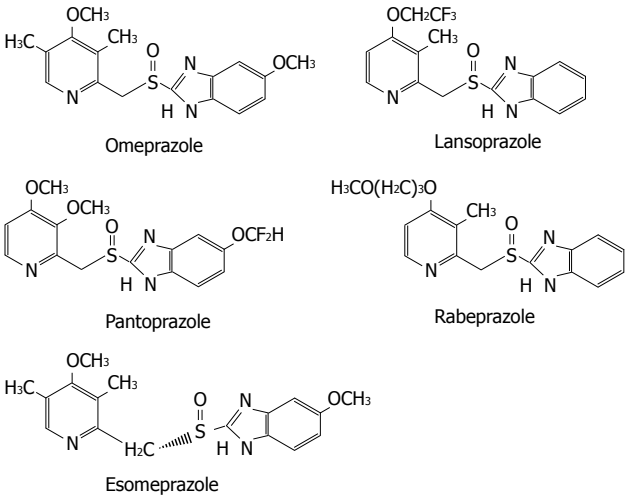

Figure 1 Chemical structures of five PPIs.

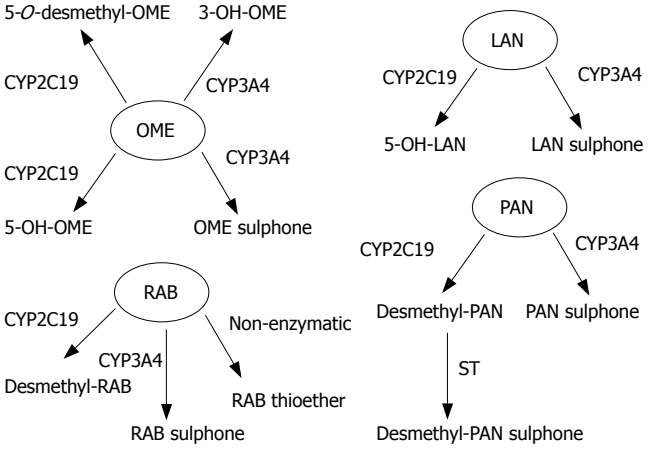

Figure 2 Metabolic differences between four PPIs (OME: omeprazole; LAN: lansoprazole; RAB: rabeprazole; PAN: Pantoprazole). Arrow thickness represents relative contribution to metabolism.

omeprazole *in vivo* is related to the genetic polymorphism of CYP1A2, dose and course of treatment[16-18]. Potential interactions between omeprazole or lansoprazole and CYP1A2 substrates with narrow therapeutic windows should be kept in mind in long-term concurrent therapy. Among these three old PPIs, pantoprazole has by far the lowest potential for interactions[19].

Rabeprazole, although metabolized partially by CYP2C19, is primarily metabolized by nonenzymatic reduction and hence genotype and modifiers of CYP2C19 have less impacts on its PK ($AUC_{po(PM)}/AUC_{po(EM)} \leqslant 1.8$) and clinical efficacy[20].

Esomeprazole is the S-enantiomer of omeprazole. Its metabolism involves CYP2C19, but to a lesser extent than omeprazole (Figure 3). Its PK is less dependent on CYP2C19 genotype ($AUC_{po(PM)}/AUC_{po(EM)}$ approximate 3.0) and hence, it has less interpatient variability in clearance than omeprazole. Moreover, esomeprazole is cleared more slowly *in vivo* and has an improved oral bioavailability, leading to the greater inhibition of gastric acid secretion compared to omeprazole[21,22].

The enantiomers of pantoprazole are differentially

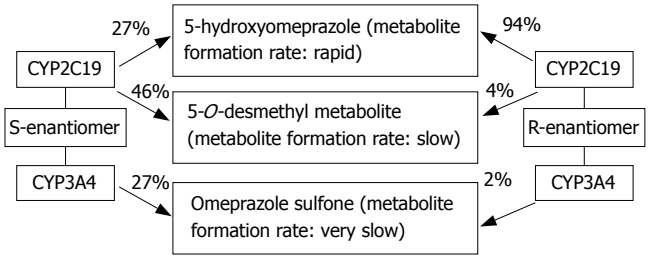

Figure 3 Stereoselective metabolism of omprazole in human.

affected by CYP2C19 genotype, such that the $AUC_{po(PM)}$/$AUC_{po(EM)}$ ratio is 11 and 2.5 for the R-(+)- and S-(−)-enantiomers, respectively[23]. Comparative clinical trial of S-(−)-pantoprazole *vs* racemic pantoprazole in the treatment of GERD has been carried out by Pai *et al*[24]. S-(−)-pantoprazole (20 mg) was found to be more effective than racemic pantoprazole (40 mg) in improving symptoms. Consequently, the use of S-(−)-pantoprazole offers both pharmacokinetic and pharmacodynamic advantages.

Many recent cost-effectiveness analyses have provided an economic basis to employ CYP2C19 genotyping prior to initiating omeprazole-, lansoprazole- or pantoprazole-based polytherapy. However, pharmacogenetic tests may be unnecessary if rabeprazole or esomeprazole based therapy are considered.

Histamine H_2-receptor antagonists

Histamine H_2-receptor antagonists are clinically applied for the treatment of gastritis, gastric and duodenal ulcers[25]. Six H_2-receptor antagonists are currently on the market: cimetidine, ranitidine, famotidine, nizatidine, ebrotidine and roxatidine acetate. Their chemical structures are depicted in Figure 4.

Martinez *et al*[26] compared the inhibitory effect of the H_2-receptor antagonists on the enzymes activities in human liver microsomes. The results were as follows: CYP1A2: cimetidine > ranitidine = ebrotidine; CYP2D6: cimetidine > ranitidine = ebrotidine; CYP3A4: ebrotidine > cimetidine > ranitidine. However, it should be cautiously considered when these *in vitro* data were extrapolated to *in vivo* situations. Firstly, cimetidine only selectively inhibits *in vivo* activities of CYP3A4 and CYP2D6[27]. For example, coadministered cimetidine increased the degree of beta-blockade of timolol (CYP2D6 substrate) ophthalmic solution and the maximum plasma concentrations of CYP3A4 substrates (e.g., midazolam and saquinavir) and disopyramide (CYP3A4 and CYP2D6 substrate)[26,28-30]. Coadministration of cimetidine 400 mg twice a day with saquinavir soft gel 1200 mg twice a day resulted in a significant increase in saquinavir $AUC_{0\text{-}24}$ (120%) and C_{max} (179%). From this view, coadministered cimetidine may be employed as a new pharmacoenhancer for boosting saquinavir for HIV infections. Beneficial effects of the inhibitory activity of cimetidine toward CYP are also used for the prevention of hepatotoxicity induced by overdoses with paracetamol, a substrate of several CYP isoenzymes which activate the drug by oxidation to the hepatotoxic metabolite N-acetyl-p-benzoquinone imine.

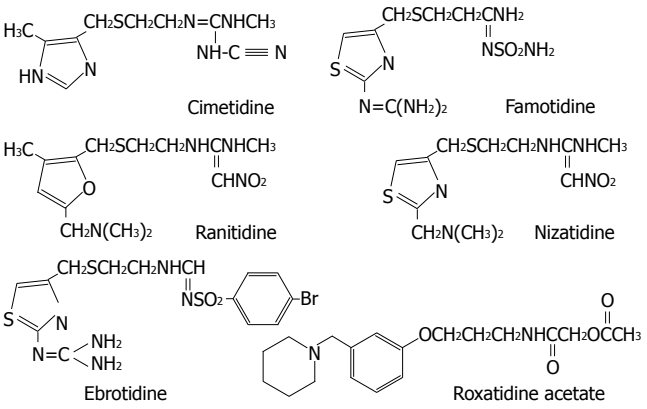

Figure 4 Chemical structures of six H_2-receptor antagonists.

Secondly, inhibition of CYP1A2 activity in humans by cimetidine has not been observed with clinical significance. In concurrent therapy of warfarin, disposition of the less potent R-warfarin (CYP1A2 substrate) was impaired. However, this interaction is likely to be of minimal clinical significance in most patients[31]. The interaction between cimetidine and theophylline was reported inconsistently. Degree of inhibition (absolute change in theophylline clearance) was closely related to route of administration, dosage, the basal theophylline clearance and smoking history[32-35]. Significant pharmacokinetic interaction between cimetidine and theophylline was not observed with low-dose cimetidine (200 mg twice daily), but with 800 mg cimetidine given once daily. Smokers or individuals with higher basal theophylline clearances had greater degree and percent of inhibition than non-smokers or individuals with lower basal theophylline clearances. It suggests that disposition of CYP1A2 substrates may still be impaired in smokers or other individuals with high CYP1A2 activities when coadministered with cimetidine. Thirdly, ebrotidine has no inhibitory effect on CYP3A4 *in vivo*, which is confirmed by lack of metabolic interaction with midazolam[26]. Overall, in contrast to cimetidine, the effects of the other H_2-receptor antagonists on CYP *in vivo* seem to have little clinical significance.

Benzamide-type gastroprokinetic agents

Benzamide-type gastroprokinetic agents (e.g., metoclopramide, cisapride, mosapride, itopride, renzapride and domperidone) are the mainstay of therapy in disorders of gastric motility such as non-ulcer dyspepsia (NUD), GERD, gastritis, diabetic gastroparesis and functional dyspepsia. Their chemical structures are depicted in Figure 5.

Among these gastroprokinetic agents, metoclopramide is predominantly metabolized by CYP2D6, thus its elimination being slow in PMs of CYP2D6 or in patients taking inhibitors of this isoform. Metoclopramide-induced acute dystonic reactions were more frequently observed in patients carrying homozygous CYP2D6 polymorphisms[36]. Meanwhile, it is also a potent inhibitor of CYP2D6 at therapeutically relevant concentrations and markedly inhibits *in vitro* codeine bioactivation[37,38]. Human pharmacokinetic interactions between metoclopramide and CYP2D6 substrates have not yet been documented.

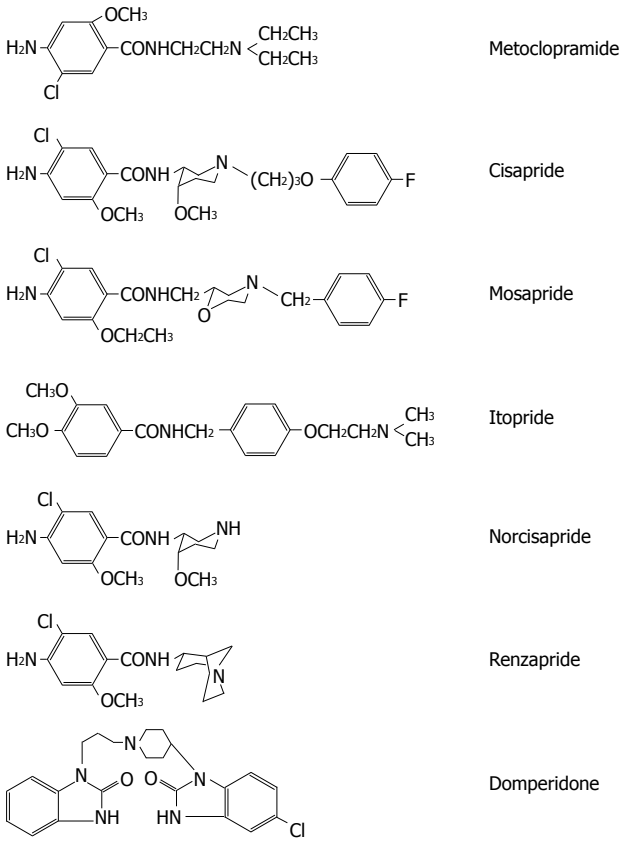

Figure 5 Chemical structures of seven benzamide-type gastroprokinetic agents.

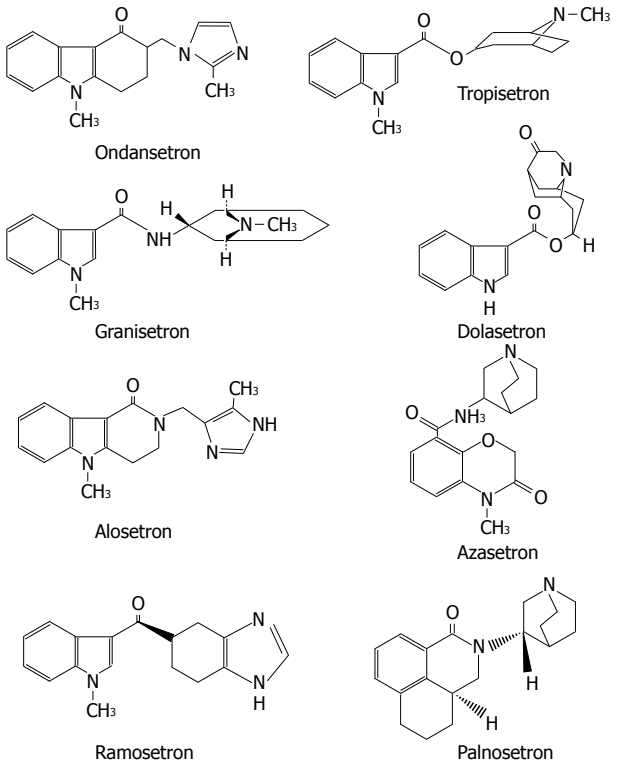

Figure 6 Chemical structures of eight setrons.

Cisapride, mosapride and domperidone, are all predominantly metabolized by CYP3A4. Their dispositions could be strongly impaired by CYP3A4 inhibitors, causing greatly elevated plasma concentrations of parent drugs[39-41]. Among these prokinetic agents, only interactions of cisapride and CYP3A4 inhibitors induce severe clinical adverse events like QT interval prolongation and/or torsades de pointe, which is responsible for the withdrawal of cisapride by FDA. However, cisapride is still on the market in some countries following restriction imposed on its usage. The most important step that can be taken to minimize the risk of cisapride-associated arrhythmias is to avoid the concomitant administration of contraindicated drugs, particularly the macrolide antibiotics (e.g., erythromycin, clarithromycin) and the azole antifungals, (e.g., itraconazole and ketoconazole).

Itopride is primarily metabolized by flavin-containing monooxygenase and its PK is unlikely influenced by CYP3A4 inhibitors[39]. Norcisapride is a major active meta-bolite of cisapride *via* CYP3A4-mediated N-dealkylation. It possesses approximately 15% of the prokinetic activity of cisapride, but has no apparent effect on myocardial conduction[42]. Compared with cisapride, norcisapride elimination does not depend on CYP, and so norcisapride does not interact with azoles or macrolides. Janssen Pharmaceutica has licensed Sepracor's patent on (+) -norcisapride, and its clinical trials are undergoing. This new compound along with mosapride, domperidone and itopride are potentially safer alternatives to cisapride in the concurrent therapy of gastroprokinetic agents with potent CYP3A4 inhibitors.

Renzapride is not metabolized by CYP. It is excreted *via* the renal route primarily unchanged. Thus, its disposition is unsusceptible to CYP modulators and it does not interfere with CYP-mediated metabolism of other drugs[43]. It is currently in clinical development for constipation-predominant irritable bowel syndrome.

Selective 5-HT$_3$ receptor antagonists

The selective 5-HT$_3$-receptor antagonists or "setrons", including ondansetron, dolasetron, tropisetron, granisetron, alosetron, azasetron, palonosetron and ramosetron (Figure 6) represent a class of antiemetics that are currently used for chemotherapy- and radiotherapy-induced, or postoperative nausea and vomiting. However, these setrons have different metabolic profiles.

Ondansetron is cleared by multiple CYP forms in humans, with no single CYP form dominating the overall metabolism. Therefore, its PK lacks bimodality and seems unchanged when ondansetron is used concomitantly with specific CYP isoenzyme inhibitors[44].

Dolasetron is rapidly reduced by carbonyl reductase to its major active metabolite hydrodolasetron, which is eliminated by multiple routes, including renal excretion and metabolism mainly by glucuronidation and hydroxylation[45]. Hence, dolasetron appears to be insusceptible to clinically significant metabolic interactions posed by drugs commonly used in chemotherapy or surgery.

Tropisetron metabolism is almost exclusively CYP2D6-dependent and the metabolites are not pharmacologically active, thus the efficacy of antiemetic treatment with tropisetron largely depends on CYP2D6 genotype. The dose of tropisetron has to be patient-tailored according to CYP2D6 genotype[46,47].

Granisetron is unique because it is not metabolized *via*

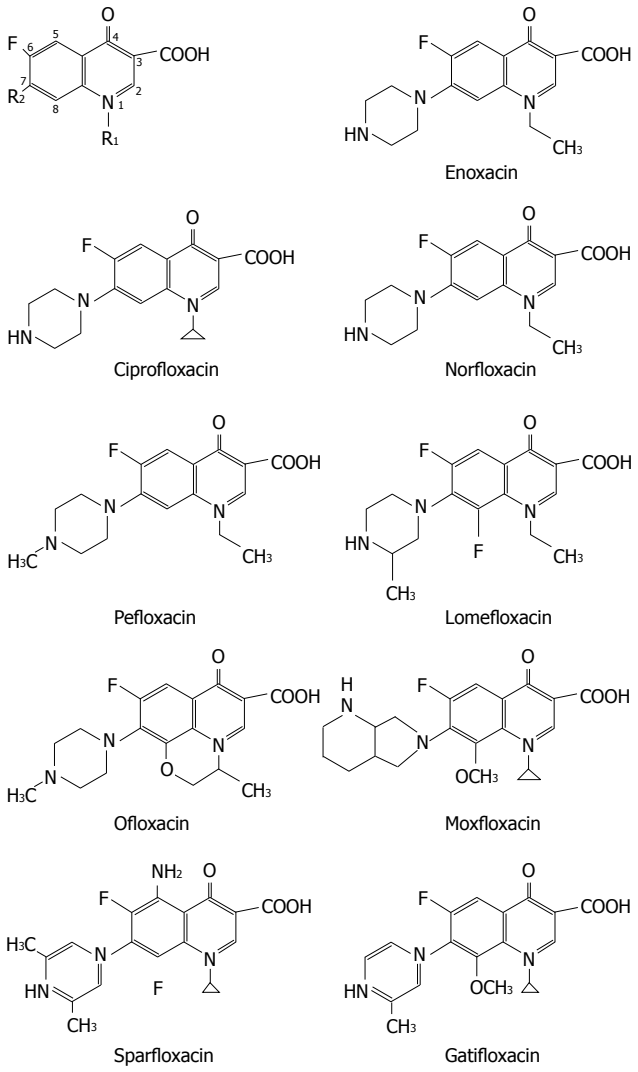

Figure 7 Chemical structures of nine fluoroquinolones.

CYP2D6. Instead, it is metabolized *via* CYP3A4, which is not subject to significant genetic polymorphism and variation in patient response. Moreover, carriers of the duplication of the CYP2D6 allele predicting ultrarapid metabolizer status had less frequent vomiting episodes in subjects receiving granisetron than patients receiving tropisetron. Use of granisetron would obviate the need for CYP2D6 genotyping and may lead to improved prophylaxis of postoperative nausea and vomiting[48-50].

Alosetron is extensively metabolized in humans. *In vivo* data suggest that CYP1A2 plays a prominent role in alosetron metabolism[51,52]. In a pharmacokinetic study, 40 healthy female subjects received fluvoxamine (a known strong inhibitor of CYP1A2) in escalating doses from 50 to 200 mg per day for 16 d, with coadministration of alosetron 1 mg on the last day. Fluvoxamine increased mean alosetron AUC by approximately 6-fold and prolonged the half-life by approximately 3-fold. Thus, concomitant administration of alosetron and strong inhibitor of CYP1A2 is contraindicated. Otherwise, dose-related side effects of alosetron such as constipation may occur more frequently[53].

Azasetron is mainly excreted in urine as the unmetabolized form (approximately 60%-70%), which is different from the fact that other setrons undergo extensive metabolism[54]. *In vitro* data suggest that azasetron does not cause clinically significant CYP-mediated drug interactions.

Palonosetron is metabolized in the liver (approximately 50%). The two primary metabolites, N-oxide-palonosetron and 6-(S)-hydroxy-palonosetron, are essentially inactive. CYP2D6 is the major enzyme of palonosetron metabolism. Clinical pharmacokinetic parameters were not significantly different between PMs and EMs of CYP2D6[55-57]. As for ramosetron, *in vitro* data with human liver microsomes showed its minimal potential to cause clinically important CYP-mediated drug interactions[58].

Fluoroquinolones

Fluoroquinolones are good choices in treatment of intestinal infections caused by sensitive bacterias. Meanwhile, fluoroquinolones-based polytherapy regimens are also used for *H pylori* infection in some occasions, especially after treatment failure in initial *H pylori* eradication[59-63].

The chemical structures of nine fluoroquinolones are listed in Figure 7. They have different CYP-mediated interaction potentials. Enoxacin, ciprofloxacin, norfloxacin and to a lesser extent pefloxacin all have inhibitory effects on metabolism of CYP1A2 substrates such as warfarin, tacrine, clozapine, tizanidine and theophylline. Ofloxacin, levofloxacin, sparfloxacin, lomexacin, gatifloxacin, sparfloxacin, lomefloxacin and moxifloxacin, are less prone to inhibit CYP1A2 and thus are alternative fluoroquinolones to patients receiving concurrent therapy of CYP1A2 substrate with narrow therapeutic window.

Moreover, ciprofloxacin and norfloxacin significantly depressed CYP3A4 in human microsomes[64]. Many case reports indicated their inhibitory effects on CYP3A4 in humans[65-69]. Clinicians should be wary of coadministration of norfloxacin or ciprofloxacin with CYP3A4 substrates with narrow therapeutic window. Table 1 lists the metabolic drug interactions related to fluoroquinolones with clinical relevance.

Macrolide antibiotics

Macrolide antibiotics are usually included in polytherapy regimen for treatment of *H pylori* gastritis[76-80]. In addition, erythromycin, clarithromycin and azithromycin all exhibit prokinetic effects and may be used in the management of gastroparesis[81-84]. For example, erythromycin therapy is effective in the treatment of patients with gastroparesis, in whom metoclopramide or domperidone was ineffective.

Macrolides can be classified into 3 groups based on the propensity of these compounds to interfere with CYP3A4[85-87]. The first group (e.g., troleandomycin, erythromycin and clarithromycin) are potent mechanism-based CYP3A4 inhibitors. Because mechanism based inhibition is an irreversible inhibition where a covalent bond is formed between a metabolite and the active site of the enzyme, destroying the enzyme's activity, so the first group of macrolides could produce drug interactions with clinical relevance. The second group (e.g., flurithromycin, midecamycin, josamycin and roxithromycin) form complexes to a lesser extent and rarely produce drug interactions. The

Table 1 Metabolic drug interactions of fluoroquinolones with clinical relevance

Polytherapy regimen	Clinical consequence	Ref
Ciprofloxacin + tizanidine	Oral ciprofloxacin (500 mg twice daily for 3 d) increased AUC (0-infinity) of tizanidine by 10-fold and Cmax by 7-fold and dangerously potentiates its hypotensive and sedative effects, mainly by inhibiting CYP1A2. Care should be exercised when tizanidine is used concomitantly with ciprofloxacin.	70
Ciprofloxacin + clozapine	Ciprofloxacin (250 mg twice daily for 7 d) can moderately increase serum concentrations of clozapine and N-desmethylclozapine in patients with schizophrenia. A probable mechanism of interaction is an inhibition of CYP1A2 by ciprofloxacin.	71
Ciprofloxacin + theophylline	The interaction between oral ciprofloxacin (500 mg twice daily for 60 h) and theophylline can be clinically significant. Inter-individual variability in the magnitude of interaction can be attributed to inter-individual differences in the level of CYP1A2 expression.	72
Ciprofloxacin + olanzapine	Ciprofloxacin treatment (250 mg twice daily for 3 d) doubled olanzapine concentrations in one patient through the inhibition of CYP1A2.	73
Ciprofloxacin + sildenafil	Ciprofloxacin significantly increased sildenafil bioavailability (above 2-fold) in healthy volunteers, mainly by CYP3A4 inhibition. Dose adjustment of sildenafil is thus necessary.	65
Ciprofloxacin + methadone	Ciprofloxacin inhibited metabolism of methadone *via* CYP1A2 and CYP3A4, and caused profound sedation, confusion, and respiratory depression	66
Ciprofloxacin + cyclosporine	Ciprofloxacin and cyclosporine may be used together safely at the recommended dosage. However, case reports have suggested a possible pharmacokinetic interaction, e.g., ciprofloxacin substantially increased cyclosporine blood levels in a patient with pure red blood cell aplasia. However, levofloxacin therapy (500 mg/d Ⅳ) did not interfere with cyclosporine blood levels and thus it could be a therapeutic alternative.	67, 68
Enoxacin + fluvoxamine	Enoxacin (200 mg/d for 11 d) significantly increased the plasma concentrations at 2, 3 h and the Cmax of fluvoxamine in healthy volunteers. Sleepiness produced by fluvoxamine increased when coadministered with enoxacin.	74
Enoxacin + theophylline	A multidose regimen of enoxacin significantly slowed the clearance of theophylline and elevated theophylline concentrations in serum. The careful monitoring of serum theophylline level and modification of theophylline dosage in patients receiving enoxacin and theophylline were recommended.	75
Norfloxacin + cyclosporine	In pediatric patients undergoing renal transplantation norfloxacin impaired cyclosporine disposition by inhibition of CYP3A4, resulting in cyclosporine dose reduction from 7.4 mg/kg per day to 4.5 mg/kg per day.	69

last group (e.g., azithromycin, dirithromycin and spiramycin) does not inhibit CYP3A4 and are unable to modify the PK behaviors of other compounds.

Metz *et al*[88] reported a potentially significant pharmacokinetic drug interaction between clarithromycin and carbamazepine in two patients with long-standing epilepsy who received omeprazole-clarithromycin therapy for *H pylori* gastritis. In both cases, clarithromycin therapy was temporally related to an increase in serum carbamazepine levels, which returned to the therapeutic range following cessation of clarithromycin therapy. If possible, erythromycin and clarithromycin should be avoided in patients taking CYP3A4 substrates such as atorvastatin, simvastatin, rifabutin, midazolam, cyclosporin, cisapride, pimozide, disopyramide, astemizole, nifedipine and carbamazepine. Azithromycin may be an alternative[89-92]. If clinical judgment suggests erythromycin and clarithromycin should be used, it is necessary to adjust dosage of CYP3A4 substrates with narrow therapeutic window (e.g., decrease the dosage of carbamazepine by 30%-50%), monitor the serum drug levels closely, and warn the patient about the signs and symptoms of toxicity.

Moreover, both erythromycin and clarithromycin are also potent inhibitors of P-glycoprotein and can significantly interfere with the PK behaviors of P-glycoprotein substrate such as digoxin. For example, a case of a clarithromycin-associated digoxin toxicity in a patient with chronic atrial fibrillation and *H pylori* infection was reported by Gooderham *et al*[93].

Azole antifungals

Azole antifungals (i.e., ketoconazole, itraconazole, fluconazole and voriconazole) may be used in treatment for fungus infections in digestive tracts. Their chemical structures are illustrated in Figure 8.

Ketoconazole is extensively metabolized into several inactive metabolites in the liver and the metabolites primarily excreted in bile[94]. Itraconazole is metabolized predominately by CYP3A4. Renal excretion of the parent drug is less than 0.03% of the dose[95]. Fluconazole is mainly excreted in urine as the unmetabolized form (approximately 80%). Accordingly, renal function is the major determinant of fluconazole PK[96]. The concurrent therapy of CYP3A4 inducers (e.g., rifampin and rifabutin) with itraconazole or ketoconazole results in poor antifungal response, thus their coadministrations are not recommended. However, fluconazole PK is less affected by CYP3A4 inducers[97], so fluconazole may be as an alternative for patients receiving comedicated CYP3A4 inducers.

Voriconazole is extensively metabolized by CYP2C19, CYP2C9 and CYP3A4. The major metabolite of voriconazole is the N-oxide, which has negligible antifungal activity. Inducers or inhibitors of these isoenzymes may increase or decrease voriconazole plasma concentrations. Coadministration of voriconazole with rifampicin, carbamazepine and phenobarbital is contraindicated. Allelic polymorphisms of CYP2C19 have been shown to be the most important determinants of the clearance of voriconazole, resulting in two phenotypes: PMs and EMs (both homozygous and heterozygous). Homozygous EMs have a two-fold lower exposure than heterozygous EMs and four-fold lower drug exposure than PMs[98-100]. Coadministration of a potent CYP3A4 inhibitor leads to a higher and prolonged exposure with voriconazole that might increase the risk of ADRs on a short-term

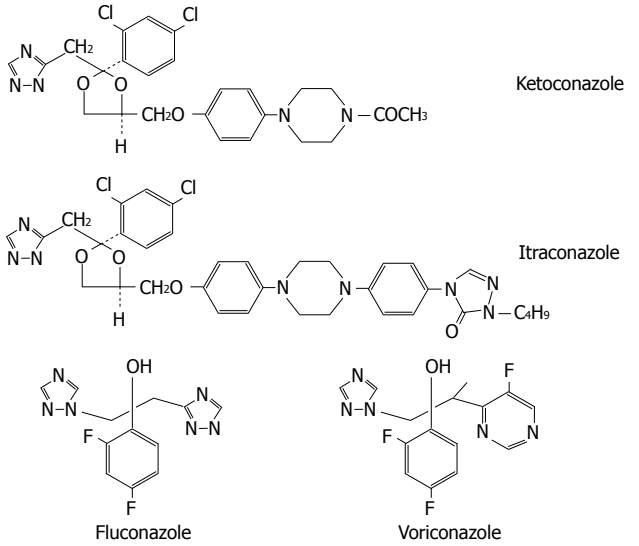

Figure 8 Chemical structures of four azole antifungals.

basis, particularly in CYP2C19 PM patients[101]. Thus, it is necessary to implement CYP2C19 genotyping prior to initiation of voriconazole therapy or therapeutic drug monitoring in the course of treatment.

Ketoconazole and itraconazole are potent inhibitors of CYP3A4. Coadministration with CYP3A4 substrates can cause clinically significant drug interactions, some of which can be life-threatening. Cisapride, oral midazolam, pimozide, quinidine, triazolam, levacetylmethadol, statins metabolized by CYP3A4 (i.e., lovastatin, simvastatin and atorvastatin), ergot alkaloids metabolized by CYP3A4 (i.e., dihydroergotamine, ergometrine, ergotamine and methylergometrine) are contraindicated with ketoconazole and itraconazole.

The potency of fluconazole as a CYP3A4 inhibitor is much lower and thus its clinical interactions with CYP3A4 substrates are of less magnitude. Doses of less than 200 mg/d are not associated with significant CYP3A4-mediated interactions. So fluconazole ($\leqslant$ 200 mg/d) is a relatively safe alternative azole antifungal when coadministered with statins metabolised by CYP3A4[102]. However, it is a potent inhibitor of CYP2C9. Coadministration of fluconazole with CYP2C9 substrates such as phenytoin, warfarin, fluvastatin and losartan leads to clinically significant drug interactions, whereas concurrent therapy of itraconazole or ketoconazole has minimal effect on PK of CYP2C9 substrates[103,104].

Voriconazole inhibits the activities of CYP2C19, CYP2C9 and CYP3A4. Thus, there is a potential for voriconazole to increase the plasma levels of substances metabolized by these CYPs. Coadministration of voriconazole with CYP3A4 substrates (e.g., terfenadine, astemizole, cisapride, quinidine and sirolimus) is contraindicated. When initiating voriconazole in patients already receiving cyclosporine or tacrolimus, it is recommended that the maintenance dosage of two immunosuppressive agents should be adjusted and that their level be carefully monitored. If patients receiving CYP2C9 substrates (e.g., warfarin, phenytoin or sulphonylureas) are treated simultaneously with voriconazole, pharmacotherapy monitoring and dosage adjustment for these drugs should be implemented accordingly.

DISCUSSION

The relationship between chemical structure and metabolic profile has been described in the above summary on seven classes of drugs for gastrointestinal diseases treatment. The underlying molecular mechanism of interactions between drug and metabolizing enzymes is complex and it determines whether the drug is a substrate or inhibitor of the specific enzyme and how far it influences the enzyme activity.

Comparative molecular field analysis (CoMFA) modelling can reveal the key molecular characteristics of CYP inhibitors. For example, both electrostatic and steric interactions were found to account for the differences in the potencies of drugs to inhibit CYP2B6. The differences in inhibitory effects of H_2-receptor antagonists on CYP enzymes may be attributed to the different ability of substituent to bind to the heme iron in CYP[105]. Cimetidine carries both the imidazole and the cyano groups which strongly bind to the heme iron and are responsible for its prominent interaction potential. In comparison to cimetidine, ranitidine has the following structural characteristics: (1) the imidazole ring is substituted with a furane ring, and (2) the side chain cyano group is substituted with a nitro group. Famotidine, nizatidine and ebrotidine all possess a thiazole nucleus instead of the imidazole ring, and the cyano-group in the side chain is substituted by aminosulfonyl- or nitro group. The affinity of binding with CYP isoenzymes is in the following order: imidazole ring (cimetidine), furane ring (ranitidine), thiazole ring (famotidine, nizatidine and ebrotidine). Roxatidine carries no imidazole group in its chemical structure, so it also has a weak inhibitory effect on CYPs.

The relationship between chemical structure of fluoroquinolone and its interaction magnitude has been determined[106, 107]. Molecular modeling studies showed that it is possible to explain the potency of the quinolones to inhibit CYP1A2 on a molecular level. The keto group, the carboxylate group, and the core nitrogen at position 1 are likely to be the most important groups for binding to the active site of CYP1A2, because of the molecular electrostatic potential in these regions. Fluoroquinolones carrying an alkylated piperazinyl moiety at the position 7 (e.g., ofloxacin, levofloxacin, sparfloxacin, lomexacin and gatifloxacin) or a bulky substituent at the position 8 (e.g., sparfloxacin, lomefloxacin, gatifloxacin and moxifloxacin), are less prone to inhibit CYP1A2 than those without corresponding substituents.

The type of CYP metabolism and degree to which an azole antifungal is metabolized are governed by a number of factors including the physiochemical properties of the drug (lipophilicity) and its PK characteristics. Because ketoconazole and itraconazole are highly lipophilic, their clearance is heavily dependent upon CYP-mediated metabolism[108]. Fluconazole, on the other hand, is relatively less lipophilic and requires less CYP-mediated

metabolism at low dosages (< 200 mg/day). Ketoconazole carries an imidazole ring, whereas itraconazole, fluconazole and voriconazole contain triazole rings. The four azole antifungals are strongly binding to hepatic microsome CYP enzymes in a Type Ⅱ manner (i.e., involving the direct ligation of an azole nitrogen with the iron atom of the haem group in the CYP enzyme), which resulted in the broad-spectrum inhibition of multiple CYP isoforms, although the relative potencies towards the various isoforms vary from drug to drug[109].

Generally, if some type of CYP isoenzyme is the most important determinants of the clearance of a drug, metabolic drug interactions can be anticipated when the drug is coadministered with inducers or inhibitors of this isoenzyme. If this drug has a relative narrow therapeutic window, drug-drug interaction may be of clinical relevance. Morever, obvious inter-individual clinical outcome may be observed in patients if a CYP isoenzyme (the determinant of the clearance of a drug) exhibts polymorphism. Under all these situations in clinical practice, clinicians and pharmcists should show abilities in medication therapy management. Careful observations are needed in using new drugs in view of few clinical experiences.

In conclusions, the metabolic profile includes the fraction of drug metabolized by CYP, CYP reaction phenotype, impact of CYP genotype on interindividual PK variability and CYP-mediated drug-drug interaction potential. Significant differences may be observed with the metabolic profiles of medications for gastrointestinal disease treatment even if they belong to the same therapeutic or structural class. Many events of severe ADRs and treatment failures were closely related to the ignorance of this respect. Clinicians should acquaint themselves with what kind of drug has less interpatient variability in clearance and whether to perform CYP genotyping prior to initiation of therapy. The relevant CYP knowledge also helps clinicians enhance the management of patients on polytherapy regimens, i.e., better anticipate or avoid a drug interaction, choose an alternative agent with lower interaction potential, and perform pharmacotherapy monitoring (e.g., monitoring clinical symptoms and alterations in laboratory values) and dosage adjustment accordingly when concurrent therapy can not be avoided.

CONMENTS

Background

Metabolism by cytochrome P450 (CYP) represents an important clearance mechanism for the majority of drugs, thus affecting their oral bioavailability, duration and intensity of pharmacological action. The metabolic profile of a drug depicts its amount metabolized by CYP, the CYP reaction phenotype, impact of the CYP genotype on interindividual pharmacokinetics variability and CYP-mediated drug-drug interaction potential. It is closely related to the three-dimensional chemical structure of drug and may exhibit significant differences among drugs within the similar therapeutic or structural class, although the efficacy of these similar drugs do not show sharp differences at the dose used clinically. Many events of severe adverse drug reactions and treatment failures are attributed to the ignorance of above issues. In order to promote rational drug use in clinical practice, it is essential to let clinicians know what kind of drug has less interpatient variability in clearance, whether to perform CYP genotyping prior to therapy and how to enhance the management of patients on polytherapy regimens from the perspective of drug metabolism.

Research frontiers

Food and Drug Administration (FDA) published guidance for *in vitro* and *in vivo* drug metabolism/drug interaction studies in the drug development process in 1999. Withdrawals of medications such as terfenadine, astemizole, cisapride, and mibefradil from the market by FDA demonstrate the relevance of metabolic drug-drug interaction profile. Some scientists tried to describe the three-dimensional quantitative structure activity relationships (QSARs) within substrates, inducers and inhibitors of CYP in recent years. There are also sporadic reports on metabolic differences in market products within the similar structural class.

Innovations and breakthroughs

This article is the first systematic summary on metabolic differences in market drug products within the similar therapeutic or structural class for gastrointestinal disease treatment.

Applications

The significance of this article is: (1) it helps doctors realize what kind of drug for gastrointestinal disease treatment has less interpatient variability in clearance and whether to perform CYP genotyping prior to therapy; (2) help doctors enhance management of patients on polytherapy regimens. Doctors will learn to better anticipate or avoid a drug interaction, choose an alternative agent with lower interaction potential, perform pharmacotherapy monitoring and adjust dosage accordingly when concurrent therapy cannot be avoided; and (3) help doctors attach equal importance to medicines for other disease treatment, and finally promote rational drug use in clinical practice.

Terminology

Drug metabolism: the process by which the drug is chemically converted in the body to a metabolite, usually through specialized enzymatic systems. Its rate is an important determinant of the duration and intensity of the pharmacological action of drugs. Cytochrome P450: the most important element of oxidative metabolism of a large number of endogenous compounds (e.g., steroids) and xenobiotics (e.g., drugs). CYP is the standard abbreviation for mammalian cytochrome P450. CYP reaction phenotype: the relative contribution of the CYP isoforms to the metabolic pathways. CYP genotyping: the process of determining the CYP genotype of an individual by molecular biology techniques. It can be used to prospectively identify individuals at risk for adverse drug reactions or therapeutic failure due to altered drug metabolism. $AUC_{po(PM)}/AUC_{po(EM)}$: the ratio of parent drug area-under-the concentration *vs*. time curve after oral dosing (AUC_{po}) derived from poor metabolizers (PM) and extensive metabolizers (EM).

Peer reviews

The review by Zhou *et al* summarizes current literature on seven classes of drugs used in the treatment of gastrointestinal diseases with respect to the clearance of these substances. It is highly interesting and may help physicians to choose an equivalent drug or drug combination in clinical practice.

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CLINICAL RESEARCH

New classification of the anatomic variations of cystic artery during laparoscopic cholecystectomy

You-Ming Ding, Bin Wang, Wei-Xing Wang, Ping Wang, Ji-Shen Yan

You-Ming Ding, Bin Wang, Wei-Xing Wang, Ping Wang, Ji-Shen Yan, Department of Hepatobiliary & Laparoscopic Surgery, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
Correspondence to: Bin Wang, Department of Hepatobiliary & Laparoscopic Surgery, Renmin Hospital of Wuhan University, Jiefang Road 238#, Wuhan 430060, Hubei Province, China. wb7112@sina.com
Telephone: +86-27-88041911-2212
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Abstract

AIM: To investigate the anatomic variations in the cystic artery by laparoscopy, and to provide a new classification system for the guidance of laparoscopic surgeons.

METHODS: Six hundred patients treated with laparoscopic cholecystectomy from June 2005 to May 2006 were studied retrospectively. The laparoscope of 30° (Stryker, American) was applied. Anatomic structures of cystic artery and conditions of Calot's triangle under laparoscope were recorded respectively.

RESULTS: Laparoscopy has revealed there are many anatomic variations of the cystic artery that occur frequently. Based on our experience with 600 laparoscopic cholecystectomies, we present a new classification of anatomic variations of the cystic artery, which can be divided into three groups: (1) Calot's triangle type, found in 513 patients (85.5%); (2) outside Calot's triangle, found in 78 patients (13%); (3) compound type, observed in 9 patients (1.5%).

CONCLUSION: Our classification of the anatomic variations of the cystic artery will be useful for decreasing uncontrollable cystic artery hemorrhage, and avoiding extrahepatic bile duct injury.

Key words: Cystic artery; Laparoscopic cholecystectomy; Bile duct injury; Calot's triangle

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INTRODUCTION

Since the advent of laparoscopic cholecystectomy in the last two decades, minimally invasive surgery has evolved through advances in videoscopic technology, instrumentation, and surgical techniques[1-14]. Currently, laparoscopic cholecystectomy is widely accepted as the gold standard in the treatment of cholelithiasis[15-18]. This new technique was initially associated with a significant increase in morbidity, and in particular, in iatrogenic biliary injury and arterial hemorrhage[19-30], perhaps due to a lack of knowledge of the laparoscopic anatomy of the gallbladder pedicle. Therefore, the laparoscopic surgeon has to deal with the new anatomical views and must be aware of the possible arterial and biliary variants.

A good knowledge of Calot's triangle is important for conventional and laparoscopic cholecystectomy. Calot's triangle is an important imaginary referent area for biliary surgery. In 1981, Rocko drew attention to possible variations in the region of Calot's triangle bordered by the cystic duct, common hepatic duct, and lower edge of the liver[31]. In 1992, Hugh suggested Calot's triangle should be renamed the hepatobiliary triangle, with the small cystic artery branches supplying the cystic duct being called Calot's arteries[32].

Cystic artery bleeding is a troublesome complication during laparoscopic cholecystectomy, which increases the rate of conversion to open surgery. If surgery is performed incorrectly, injury to the extrahepatic bile duct or intra-abdominal organs is inevitable. The reported incidence of conversion to open surgery because of blood vessel injuries is approximately 0%-1.9% during laparoscopic cholecystectomy[33], and its mortality is about 0.02%[34]. Safe laparoscopic cholecystectomy demands a good knowledge of the anatomy of the cystic artery and its variations.

The cystic artery has many possible origins, with the right hepatic artery being the most common[35]. The anatomy with respect to the cystic artery between laparoscopic cholecystectomy and open cholecystectomy is different. We investigated the appearance of the cystic artery during laparoscopic cholecystectomy, and proposed a new classification system for the cystic artery during laparoscopic cholecystectomy, according to our practical experience.

MATERIALS AND METHODS

Between June 2005 and May 2006, we undertook a retrospective evaluation of 600 non-emergency patients, 232 men and 368 women, who underwent laparoscopic

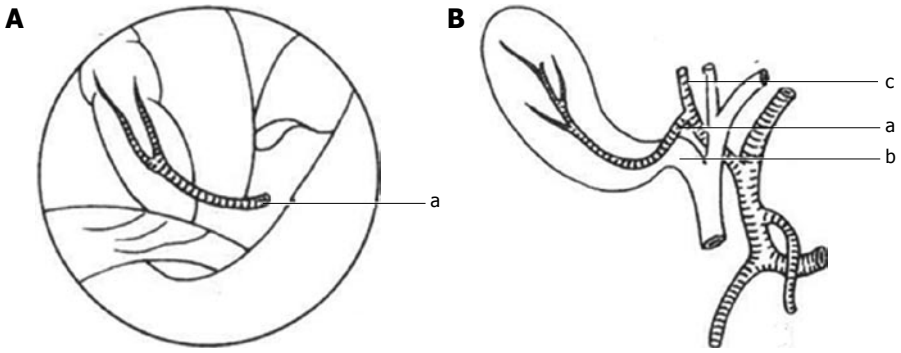


Figure 1 Classical single cystic artery. **A**: Laparoscopic visualization; **B**: Conventional visualization. a: Cystic artery; b: Cystic duct; c: Right hepatic artery.

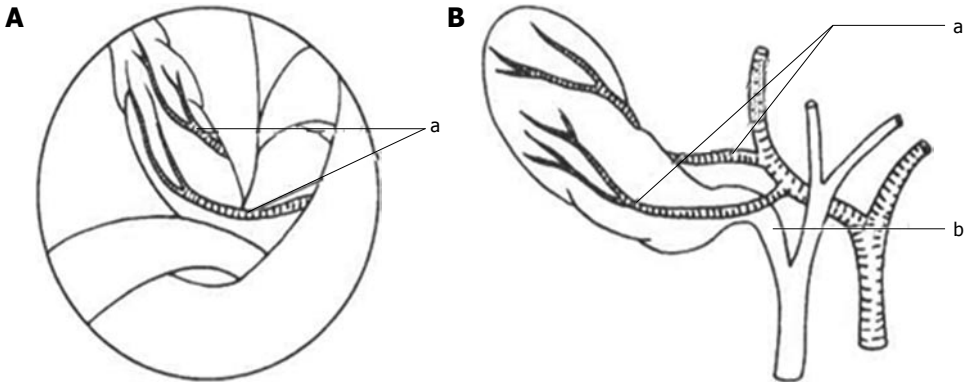


Figure 2 Double cystic artery. **A**: Laparoscopic visualization; **B**: Conventional visualization. a: Cystic artery; b: Cystic duct.

cholecystectomy for different gallbladder diseases, including 530 with cholecystitis and gallstones, and 70 with gallbladder polyps. All of the patients were examined with ultrasound before surgery.

Laparoscopic cholecystectomy was carried out under general anesthesia using the four ports technique. The information of Calot's triangle and distribution of cystic artery on endoscopic visualization was recorded respectively. A laparoscope (Stryker, USA) at 30° tilt angle was used. The anatomical structures were viewed on a three-dimensional video monitor.

RESULTS

Based on our laparoscopic observations, we classified cystic artery anatomy into three groups.

Group I

Group I represents the Calot's triangle type, in which the cystic artery passes through Calot's triangle. The anatomic location of the cystic artery can be found ahead or behind of the cystic duct, and in hepatoduodenal ligament, under laparoscopic observation. This is the most common type and has been reported in about 80%-96% of cases in previous studies[35,36]. We observed this type in 513 of the 600 patients (85.5%). Group I is further subdivided into two subtypes, as follows.

Classical single cystic artery: The cystic artery originates from the right hepatic artery within Calot's triangle. When approaching the gallbladder, the artery is divided into deep and superficial branches at the neck of the gallbladder. The superficial branch proceeds along the left side of the gallbladder. The deep branch runs through the connective tissues between the gallbladder and liver parenchyma. The deep branch gives rise to tiny branches to supply the gallbladder, which anastomose with the superficial branches. This type of cystic artery is laterally positioned from the cystic duct within Calot's triangle during open cholecystectomy, whereas during laparoscopic cholecystectomy it is just behind and slightly deeper than the cystic duct. According to the literature, this type is found in 70%-80% of cases[32,35]. In our study it was recorded in 440 of 600 patients (73.3%) (Figure 1).

Suzuki *et al* has reported a special example of this type. A single cystic artery originates from the right hepatic artery and then hooks around the cystic duct from behind and reappears at the peritoneal surface near the neck of the gallbladder. They named this the cystic artery syndrome[37]. We did not find any examples of this type in our study.

Double cystic artery: The cystic artery also originates from the right hepatic artery, while it divides into the anterior and posterior branches at their cystic artery origin. Congenital absence of the deep branch signifies the existence of another cystic artery, which is occasionally detected by subsequent bleeding control. The posterior cystic artery is very delicate in some cases and is often cut by electrocoagulation during dissection. A double cystic artery has previously been found in 15%-25% of patients[32]. During our laparoscopic cholecystectomy we recorded 73 patients (12.2%) with double cystic artery (Figure 2).

Group II

Cystic artery approaches the gallbladder outside Calot's triangle and cannot be observed within the triangle by

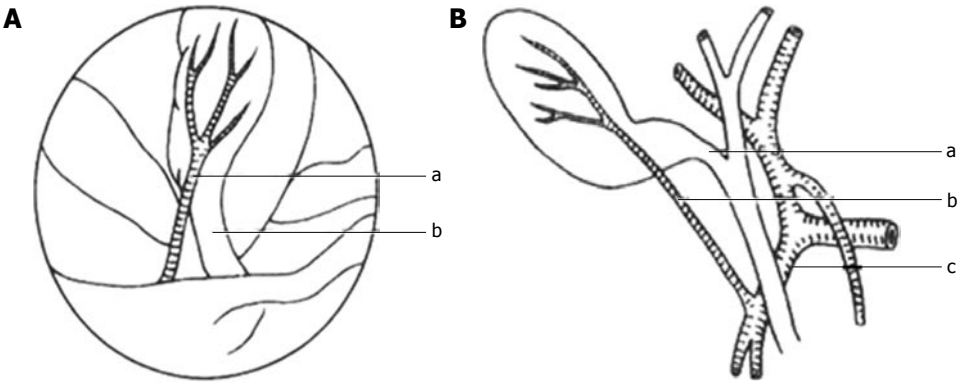
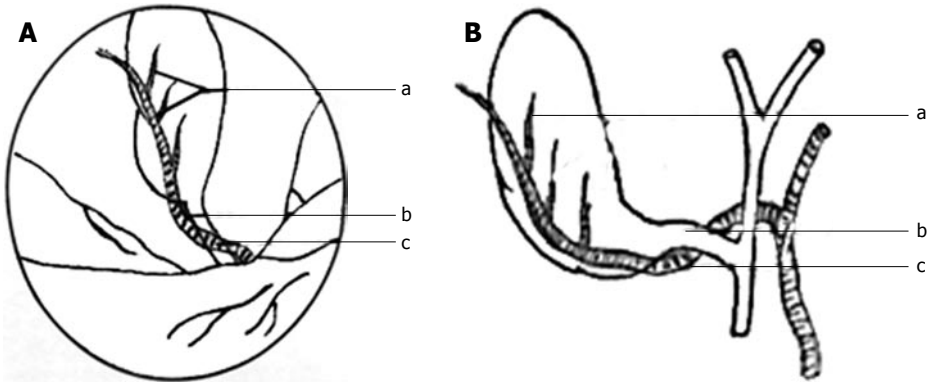


Figure 3 Cystic artery originating from gastroduodenal artery. **A**: Laparoscopic visualization; **B**: Conventional visualization. a: Cystic artery; b: Cystic duct; c: Gastroduodenal artery.



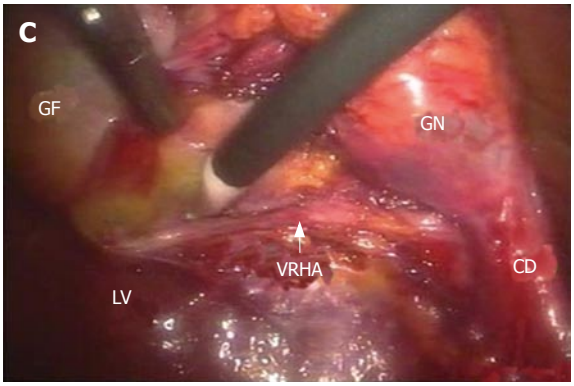


Figure 4 Cystic artery originating from variant right hepatic artery. **A**: Laparoscopic visualization; **B**: Conventional visualization; **C**: Video in operation. a: Cystic artery; b: Cystic duct; c: Variant right hepatic artery; GF: Fundus of gallbladder; CD: Cystic duct; GN: Neck of gallbladder; LV: Liver; VRHA: Variant right hepatic artery.

laparoscopy during dissection. We found 78 patients (13%) in group II during laparoscopic cholecystectomy. This group includes the following four subgroups.

Cystic artery originating from gastroduodenal artery: This type of cystic artery is also called low-lying cystic artery, which does not pass through Calot's triangle but approaches the gallbladder beyond it. In conventional open cholecystectomy it is seen as inferior to the cystic duct, while it usually localizes superficially and anterior to the cystic duct from a laparoscopic viewpoint. Its terminal segment as it approaches the gallbladder is important for laparoscopic surgeons. Because it not only must be manipulated at first, but it is also susceptible to injury and hemorrhage during dissection of the peritoneal folds that connect the hepatoduodenal ligament to Hartman's pouch of the gallbladder or to the cystic duct. This anatomic variation was found in 45 patients (7.5%) in our study (Figure 3).

Cystic artery originating from the variant right hepatic artery: Anatomic variation of the right hepatic artery usually originates from the superior mesenteric artery or aorta[32,38]. It enters Calot's triangle behind the portal vein, and runs parallel to the cystic duct on its passage through the triangle. It can be completely covered by the cystic duct of the gallbladder[32]. We found a very interesting type of right hepatic artery variant. This artery has a long course, approaching the gallbladder deep at its neck, and then passing between the gallbladder and liver parenchyma, extending along the deep right side of the gallbladder, and finally entering the liver parenchyma near the right-lateral side of the gallbladder fundus. It yields multiple small branches to supply the gallbladder at its body, and it is often completely covered by the gallbladder. We should be cautious of this right hepatic artery variation. From the laparoscopic viewpoint it looks like a single large artery. This anatomic variation was found in 18 patients (3%) in our study (Figure 4).

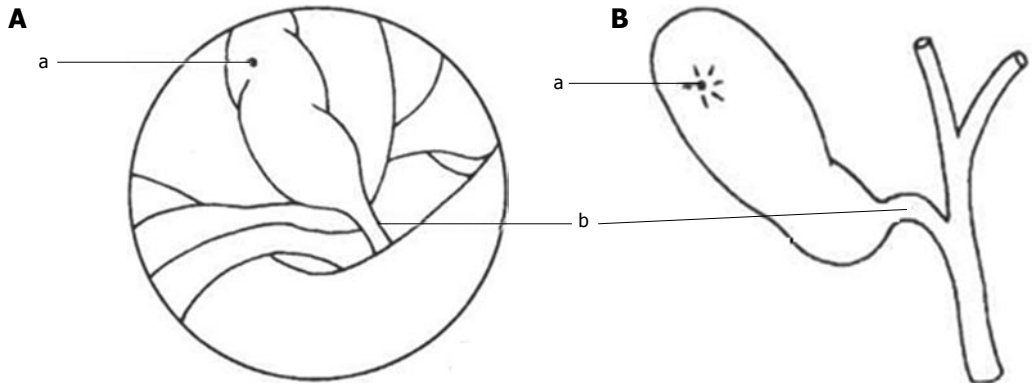


Figure 5 Cystic artery arising from hepatic parenchyma. **A**: Laparoscopic visualization; **B**: Conventional visualization. a: Cystic artery; b: Cystic duct.

Variant right hepatic artery has been shown to have a prevalence of approximate 4%-15%[37,39].

Cystic artery originating directly from the liver parenchyma: This cystic artery pierces the hepatic parenchyma approaching the bladder from the gallbladder bed. It usually situates in the right lateral of the border of gallbladder body and bottom. However, a few are situated in the center of the gallbladder bed or situated left lateral of gallbladder bottom. No other arteries are found within Calot's triangle. This anatomic variation of the cystic artery is not observed until bleeding and is caused by dissection of the gallbladder fundus. It is difficult to explore and requires careful dissection. We found it in 15 patients (2.5%) (Figure 5).

Cystic artery originating from the left hepatic artery: The cystic artery occasionally originates from the left hepatic artery, passes through the liver parenchyma, and reaches the middle of the gallbladder body, at which point it bifurcates into ascending and descending branches. This has a prevalence of 1%[39]. However, we did not find this type of variant cystic artery.

Group Ⅲ

This group has more than one blood supply. We named it the compound cystic artery type. The cystic arteries exist not only in Calot's triangle, but also outside it. We found that nine patients (1.5%) belonged to this group. Five of these patients (0.8%) had a normal single cystic artery in Calot's triangle, and an artery extending along the cystic duct but posterior to it, and some small arteries that passed immediately from the liver parenchyma to the gallbladder. Three of the nine patients (0.5%) had another cystic artery superficial to the cystic duct in addition to the normal cystic artery. Finally, one patient (0.17%) had multiple cystic arteries, including the double cystic artery in Calot's triangle, and one of the arteries crossed anterior to the common bile duct, while another was situated on the right side of the border of the gallbladder body and fundus.

DISCUSSION

Anatomic variations in and around Calot's triangle are frequent (biliary tree, cystic artery)[40,41], and we found them in 20%-50% of patients. Therefore, careful blunt dissection of Calot's triangle is necessary for both conventional and laparoscopic cholecystectomy.

Since the introduction of laparoscopic gallbladder surgery, surgeons have been very interested in gallbladder vascularization. A great number of papers on this issue have been published[36,42-44], although some vagueness still exists, because of the diversity of data and classification. There have been relatively few reports on the laparoscopic anatomy of the hepatobiliary triangle, especially of the cystic artery.

It is important for every laparoscopic surgeon to be familiar with the anatomic variations in the extrahepatic biliary tree and those of the arterial supply of the gallbladder. The possible anatomic position and variations of the cystic artery are difficult to establish before surgery. They were only identified during disconnection of Calot's triangle and the gallbladder. The laparoscopic anatomy of the cystic artery can be considered as a precondition for performing safe laparoscopic procedures. The variations of cystic artery often make surgeons recognize an error, causing them to abscise incorrectly and, subsequently, leading to a hemorrhage. When hemorrhage cannot be controlled, conversion to open cholecystectomy is inevitable.

The position of the cystic artery appears differently during laparoscopic and conventional cholecystectomy for the following reasons. (1) Under laparoscopy, as the gallbladder fundus is pulled, the liver is moved upward, thereby opening the subhepatic space. (2) By pulling Hartman's pouch downward, the anterior aspect of Calot's triangle is presented. On the contrary, by pulling Hartman's pouch upward, the posterior aspect of Calot's triangle is clearly exposed. (3) Better transparency and visualization under laparoscopy facilitates recognition of cystic artery variation by the surgeon.

Previous studies have contained fewer reports on the laparoscopic classification of the cystic artery. Some have divided the cystic artery into low-lying cystic artery and cystic artery originating from variant right hepatic artery. Balija classified cystic artery variations into two groups. Group I comprises five variations of the cystic artery within the hepatobiliary triangle: (a) normal position; (b) frontal cystic artery; (c) backside; (d) multiple; and (e) short cystic artery that arises from an aberrant right hepatic artery. Group Ⅱ consists of variations of the cystic artery that approaches the gallbladder beyond the hepatobiliary

triangle: (a) low-lying; (b) transhepatic; and (c) recurrent cystic artery[36]. Ignjatovic has divided the cystic artery into three types in minimally invasive surgical procedures: type 1 shows normal anatomy; type 2 more than one artery in Calot's triangle; and type 3 no artery in Calot's triangle[45]. However, none of the above classifications satisfies the practical needs of laparoscopic surgery. Based on our experience, the anatomic variations of the cystic artery can be classified into three groups. Group I showed the cystic artery passing within Calot's triangle. It included two types: (1) single cystic artery, found in 440 patients (73.3%); and (2) double cystic artery, observed in 73 patients (12.2%). Group Ⅱ showed the cystic artery situated outside Calot's triangle. This group included four variations: (1) cystic artery originating from the gastroduodenal artery, found in 45 patients (7.5%); (2) cystic artery originating from the variant right hepatic artery, found in 18 patients (3%); (3) cystic artery directly arising from the liver parenchyma, observed in 15 patients (2.5%); and (4) cystic artery originating from the left hepatic artery. Group Ⅲ had a compound appearance, with the variant cystic artery situated not only within Calot's triangle, but also outside it. This classification of cystic artery can help surgeons understand the cystic artery more thoroughly, and may be more practical to use in real operations.

Variations in the cystic artery are miscellaneous, and we must be cautious during the performance of laparoscopic cholecystectomy. Our laparoscopic classification of the cystic artery is very useful for dissection of Calot's triangle, reduces uncontrollable cystic artery hemorrhage, and may be advantageous for avoiding extrahepatic bile duct injury.

COMMENTS

Background

Uncontrolled bleeding from the cystic artery and its branches is a serious problem that may increase the risk of intraoperative injury to vital vascular and biliary structures. Anatomic variations of the cystic artery are frequent and can differ in origin, position and number. We investigated 600 cases treated with laparoscopic cholecystectomy and analyzed the anatomic structure of the cystic arteries under laparoscopic observation. The cystic artery variations were classified into three groups: (1) Calot's triangle, (2) outside Calot's triangle, and (3) compound type. These will be helpful to laparoscopic surgeons.

Research frontiers

Laparoscopic cholecystectomy is widely accepted as the gold standard in the treatment of gallstone disease. However, the incidence of bile duct injury caused by this procedure is more than that caused by conventional cholecystectomy. The main areas of research in laparoscopic cholecystectomy are as follows: (1) biliary injury during laparoscopic cholecystectomy; (2) anatomic characteristics of the cystic artery; (3) intraoperative bleeding during laparoscopic cholecystectomy; (4) cholecystectomy techniques.

Innovations and breakthroughs

There are few reports concerning classification of the cystic artery during laparoscopic cholecystectomy. Some authors have classified it into two groups: Group Ⅰ comprises variations of the cystic artery within the hepatobiliary triangle; and Group Ⅱ comprises variations of the cystic artery that approach the gallbladder beyond the hepatobiliary triangle. Other authors have divided cystic artery variations into three types: type 1, normal anatomy; type 2, more than one artery in Calot's triangle; and type 3, no artery in Calot's triangle. We present a new classification of cystic artery variations based on three groups. Group I shows the cystic artery passing within the Calot's triangle. This includes two types: (1) single cystic artery, and (2) double cystic artery. Group Ⅱ shows the cystic artery situated outside Calot's triangle. This group includes four variations: (1) cystic artery originating from the gastroduodenal artery; (2) cystic artery originating from the variant right hepatic artery; (3) cystic artery arising directly from the liver parenchyma; (4) cystic artery originating from the left hepatic artery. Group Ⅲ shows the cystic artery is compound in nature; the variant cystic artery was situated not only within Calot's triangle, but also outside it. Furthermore, we found a new variant right hepatic artery (Figure 4), which has not been reported before.

Peer review

The authors introduce a new classification of cystic artery variations during laparoscopic cholecystectomy. Their system is different from those reported previously. The new system divides the anatomic variations of the cystic artery into three groups according to the position of the cystic artery relative to Calot's triangle, as seen by laparoscopy. The classification system should be useful to laparoscopic surgeons, and help reduce incidences of bile duct injury and intraoperative bleeding during laparoscopic cholecystectomy.

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RAPID COMMUNICATION

Constitutive androstane receptor agonist, TCPOBOP, attenuates steatohepatitis in the methionine choline-deficient diet-fed mouse

Edwina S Baskin-Bey, Akira Anan, Hajime Isomoto, Steven F Bronk, Gregory J Gores

Edwina S Baskin-Bey, Akira Anan, Hajime Isomoto, Steven F Bronk, Gregory J Gores, Department of Medicine, Division of Gastroenterology and Hepatology, Miles and Shirely Fitterman Center for Digestive Diseases, Mayo Clinic College of Medicine, Rochester, MN 55905, United States
Supported by NIH grants T32 DK07198-26 (to ESB) and DK41876 (to GJG), and the Palumbo and Mayo Foundation
Correspondence to: Gregory J Gores, MD, Professor of Medicine, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, Minnesota 55905,
United States. gores.gregory@mayo.edu
Telephone: +1-507-2840686 Fax: +1-507-2840762
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Abstract

AIM: To ascertain whether constitutive androstane receptor (CAR) activation by 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP) modulates steatohepatitis in the methionine choline-deficient (MCD) diet-fed animal.

METHODS: C57/BL6 wild-type mice were fed the MCD or standard diet for 2 wk and were treated with either the CAR agonist, TCPOBOP, or the CAR inverse agonist, androstanol.

RESULTS: Expression of CYP2B10 and CYP3A11, known CAR target genes, increased 30-fold and 45-fold, respectively, in TCPOBOP-treated mice fed the MCD diet. TCPOBOP treatment reduced hepatic steatosis (44.6 ± 5.4% *vs* 30.4 ± 4.5%, $P < 0.05$) and serum triglyceride levels (48 ± 8 *vs* 20 ± 1 mg/dL, $P < 0.05$) in MCD diet-fed mice as compared with the standard diet-fed mice. This reduction in hepatic steatosis was accompanied by an increase in enzymes involved in fatty acid microsomal ω-oxidation and peroxisomal β-oxidation, namely CYP4A10, LPBE, and 3-ketoacyl-CoA thiolase. The reduction in steatosis was also accompanied by a reduction in liver cell apoptosis and inflammation. In contrast, androstanol was without effect on any of the above parameters.

CONCLUSION: CAR activation stimulates induction of genes involved in fatty acid oxidation, and ameliorates hepatic steatosis, apoptosis and inflammation.

Key words: Apoptosis; CYP4A; Fatty acid oxidation; Inflammation

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INTRODUCTION

Hepatic steatosis or fatty infiltration of the liver has reached epidemic proportions in Western Society. Approximately 30 million adults in the United States have hepatic steatosis, and a subset of these individuals will develop accompanying hepatic inflammation referred to as steatohepatitis[1]. If the steatohepatitis is not associated with significant alcohol intake, it is commonly referred to as nonalcoholic steatohepatitis (NASH). Unfortunately, NASH can progress to cirrhosis and chronic liver failure with considerable morbidity and mortality[2]. Treatment options for NASH are limited and, therefore, there is an unmet need for the pharmacologic treatment of this liver disease.

Although the precise etiopathogenesis of NASH remains to be defined, it is a disease due to perturbations of intermediary fat metabolism. In hepatic steatosis, an excess of non-esterified fatty acids are released from peripheral tissues into the serum[3]. These excess serum-free fatty acids are cleared by the liver where they are esterified and accumulate as neutral fat. The formation of neutral fat is presumably due to a limited capacity to oxidize excess fatty acids. Mechanisms to enhance hepatic fatty acid oxidation are, therefore, a potential strategy to protect the liver from hepatic steatosis. Hepatic fatty acid oxidation occurs by three pathways[4]. β-oxidation is a predominant pathway, which occurs in the mitochondria, and is rate-regulated by carnitine palmitoyltransferase (CPT1) and the mitochondrial trifunctional protein (MPT) complex. Peroxisomal β-oxidation oxidation occurs within peroxisomes and is rate-limited by the peroxisomal L-bifunctional enzyme (L-PBE), acetyl-COA oxidase (ACO), and urate oxidase (UO). The third

pathway is ω-oxidation which occurs in the endoplasmic reticulum. This pathway is dependent upon expression of the cytochrome enzymes CYP4A10 and CYP4A14. Stimulation of these pathways either individually or collectively could help remove excess free fatty acids from the liver and attenuate NASH.

Nuclear receptors are a family of transcription factors, which regulate metabolism of endo- and xenobiotic compounds[5]. In particular, the constitutive androstane receptor (CAR), which is highly expressed in the liver, is a biosensor for endo- and xenobiotic compounds, such as toxic bile acids[6-8] and steroids[9]. CAR mediates the induction of detoxifying enzymes by the widely used antiepileptic drug phenobarbital in humans and by the potent synthetic inducer, 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP) in mice. Once stimulated, CAR increases expression of *CYP2B* genes in the mouse[7,8,10] and human[11]. From a teleological perspective, this nuclear receptor can be viewed as a general hepatoprotective response system, as it detoxifies potentially injurious endo- and xenobiotics. In addition, it serves as a hepatic mitogen and as an anti-apoptotic agent by increasing transcriptional expression of the anti-apoptotic protein, Mcl-1[12,13]. These composite effects eliminate toxins, enlarge the liver[14], and render it resistant to liver injury-all hepatoprotective responses. Whether CAR also protects the liver from injurious endobiotics, such as free fatty acids, is less clear but a viable concept.

Based on the above concepts, we postulated that CAR would protect the liver from the development of steatohepatitis by enhancing processes involved in fatty acid oxidation. Thus, the overall objective of the current study was to ascertain whether CAR activation by TCPOBOP modulates steatohepatitis induced by the methionine choline-deficient diet, a well known murine model of NASH[15]. Two fundamental questions were formulated. In TCPOBOP-treated MCD diet-fed mice: (1) Does CAR activation modify steatosis, and if so, does it alter expression of genes involved in fatty acid oxidation? and (2) does CAR alter apoptosis and inflammation in this model of NASH? The results indicate that CAR activation can stimulate an induction of genes involved in fatty acid oxidation, ameliorating hepatic steatosis, apoptosis and inflammation. These observations suggest CAR stimulation renders the liver resistant to MCD diet-mediated steatohepatitis. An understanding of these mechanisms may allow the development of CAR agonists as therapeutic strategies for NASH.

MATERIALS AND METHODS

Animal models

The care and use of the animals for this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). C57/BL wild-type mice (Jackson laboratories, Bar Harbor, ME), weighing 20-25 g, were fed a methionine choline-deficient (MCD) diet (Harland Tech Lad, Madison, WI) for 2, 3 or 4 wk. This diet rapidly induces steatosis and steatohepatitis in rodents[16]. The mice were maintained in a temperature-controlled, pathogen-free environment and fed a standard rodent chow diet and water ad libitum. To assess the effect of CAR modulation, mice were intraperitoneally (ip) injected with either vehicle (corn oil), 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) (Sigma-Aldrich, St Louis, MO) (3 g/kg) daily for the first 3 d[7] at the starting of the MCD diet, or 5β-androstan-3β-ol (androstanol) (a CAR inverse agonist) (Steraloids, Newport, RI) (100 mg/kg) daily for 3 d at the starting of weeks one and two of the MCD diet (total of 6 injections). Androstanol was used as negative control for CAR target genes. At selected times, the animals were anesthetized with ether, and a hepatectomy was performed prior to euthanasia *via* exsanguination. Blood was drawn *via* the portal vein to examine triglyceride levels. Liver tissue sections were placed in fixative for subsequent microscopic analyses. Liver sections were also subjected to RNA extraction using the Trizol Reagent (Invitrogen, Carlsbad, CA).

Oil red O staining and fat quantification

Liver sections were cut into a thickness of 20 μm in a cryostat, air dried, and then stained with oil red O as per standard techniques[17]. Slides were then viewed under microscopy (Axioplan 2, Carl Zeiss, Inc. Oberkochen, Germany). Digital pictures were captured to quantitate percent fat (red color in area/field area × 100) of digital photomicrographs as described previously[18].

Histology, TUNEL assay and immunohistochemical identification of activated caspases 3/7

Histology, TUNEL assay, and immunohistochemical analysis for activated caspases 3/7 was performed as previously described by us[18]. To accurately quantitate TUNEL-positive and caspase 3/7-positive cells, slides were examined by digital image analysis to quantitate the percent fluorescence/field area of digital photomicrographs as described previously[13].

Measurement of Leukotriene B4

LTB4 was quantitated using a specific ELISA kit (R&D systems) according to the manufacturer's instructions[19].

Immunohistochemistry for CD68

Unstained slides of liver tissue specimens were deparaffinized and hydrated. Antigen retrieval was performed using EDTA (1 mmol/L, pH 8.0). Slides were placed in a vegetable steamer for 40 min at 97℃, followed by a cooling for 2 min. Thereafter, the catalyzed signal amplification system (DAKO, Carpinteria, CA) was used according to manufacturer's instructions. CD68 immunostaining was performed as previously described by us[20].

Real time-polymerase chain reaction (RT-PCR)

Total RNA was obtained from whole liver as previously described by us[21]. After the reverse transcription reaction, the cDNA template was amplified by PCR with Taq polymerase (Invitrogen) and mRNA was quantitated for acetyl-CoA oxidase (ACO), carnitine palmitoyltransferase (CPT1); peroxisomal 3-ketoacyl-CoA thiolase (ketoacyl thiolase), L-peroxisomal bifunctional enzyme (L-PBE), mitochondrial trifunctional protein (MTP) subunits alpha

Table 1 Primer sequences used for RT-PCR

Target	Primer sequence	Product size (bp)
ACO	F 5'-GAACTCCAGATAATTGGCACCTA-3'	75
	R 5'-AGTGGTTTCCAAGCCTCGAA-3'	
CPT-1b	F 5'-ATCATGTATCGCCGCAAACT-3'	85
	R 5'-CCATCTGGTAGGAGCACATGG-3'	
CYP2B10	F 5'-CAA TGGGGA ACG TTG GAA GA-3'	176
	R 5'-TGATGCACTGGAAGAGGA AC-3'	
CYP3A11	F 5'-CTCAATGGTGTGTATATCCCC-3'	423
	R 5'-CCGATGTTCTTAGACACTGCC-3'	
CYP4A10	F 5'-AGTGTCTCTGCTCTAAGCC-3'	180
	R 5'-CCCAAAGAACCAGTGAAA-3'	
Ketoacyl thiolase	F 5'-GCATCCCAGAGACTGTACCTTT-3'	202
	R 5'-GTCTCTGGCCTTCTCGTTCT-3'	
L-PBE	F 5'-TGGCTCTTGGAGGAGGACTAG A-3'	125
	R 5'-AAGCTGCGTTCCTCTTGCA-3'	
MPT-α	F 5'-GGCAGTCTCAGTCGCTTCTC-3'	240
	R 5'-GCACTCCTGATTTGGTCGTT-3'	
MPT-β	F 5'-AGAGCTGCACTTTCGGGTTT-3'	202
	R 5'-CTGTGGTCATGGCTTGGTTT-3'	
PPAR-α	F 5'-GTACGGTGT GTATGAAGCCATCTT-3'	76
	R 5'-GCCGTACGCGATCAGCAT-3'	
Urate oxidase	F 5'-ACCTCCCGTCATTCACTCT-3'	438
	R 5'-ACTGTCCCTGTTATTTTGCC-3'	

ACO: Acetyl-CoA oxidase; CPT1: Carnitine palmitoyltransferase; Ketoacyl thiolase: Peroxisomal 3-ketoacyl-CoA thiolase; L-PBE: L-peroxisomal bifunctional enzyme; MTP: Mitochondrial trifunctional protein subunits alpha and beta; PPAR-α: Peroxisome proliferators-activated receptor-alpha.

and bet, peroxisome proliferators-activated receptor-alpha (PPAR-α) as previously described[22]. Primers used are listed in Table 1. 18S primers (Ambion, Austin TX) were used as a control for RNA isolation and integrity. All PCR products were confirmed by gel electrophoresis. Real-time PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBR green as the fluorophore (Molecular probes). The results were expressed as a ratio of product copies per milliliter to copies per milliliter of housekeeping gene 18S from the same RNA (respective cDNA) sample and PCR run.

Statistical analysis

All data represent at least three independent experiments and are expressed as mean ± SE of the mean. Differences between groups were compared using Student *t*-tests and one-way analysis of variance with post hoc Dunnett test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Stimulating CAR target genes in the MCD diet-fed mouse by TCPOBOP

CYP2B10 and CYP3A11 have been identified as CAR target genes[10,23,24]. Therefore, to determine if TCPOBOP activates CAR in the MCD diet-fed mouse, expression of these genes was examined. When TCPOBOP treatment was administered to the MCD diet-fed mice, expressions of both CYP2B10 and CYP3A11 increased (Figure 1A and B). We also observed 45-fold and 30-fold elevations in CYP2B10 mRNA level in TCPOBOP-treated standard

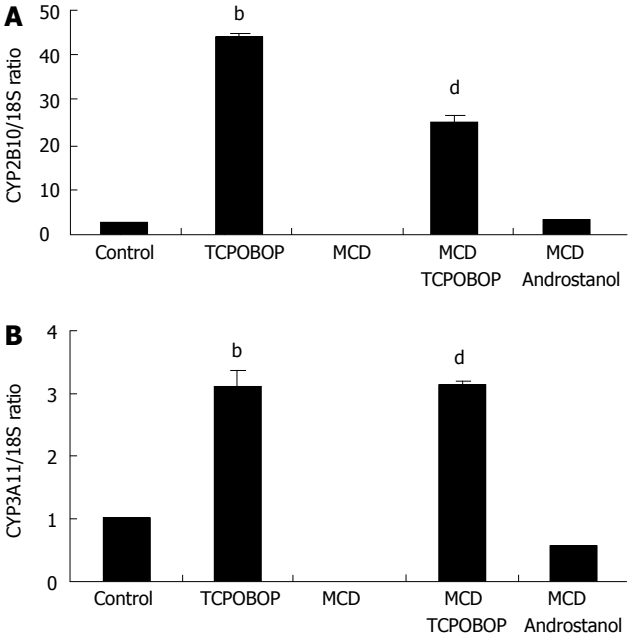

Figure 1 Over-expression of CAR target genes in MCD diet-fed mice treated with TCPOBOP. CAR activation was assessed by measuring CYP2B10 and CYP3A11 (CAR target genes) expression in whole liver from vehicle-treated and TCPOBOP-treated chow-fed and MCD diet-fed mice. Expression was measured by real-time PCR and normalized as a ratio using 18S mRNA as housekeeping genes. A value of 1 for this ratio was arbitrarily assigned to the data obtained from vehicle-treated $CAR^{+/+}$ mice. (**A**) CYP2B10 and (**B**) CYP3A11 mRNA expressions were increased in TCPOBOP-treated (3 mg/kg ip for 3 d) chow-fed mice ($^bP < 0.01$ for CYP2B10 and CYP3A11 compared to control) and MCD diet-fed mice ($^dP < 0.01$ for CYP2B10 and CYP3A11 compared to untreated). This phenomenon was abated by treatment with a CAR inhibitor androstanol (100 mg/kg ip) daily for 3 d at the starting of weeks one and two of the MCD diet (total of 6 injections), ($n = 5$ in each group).

diet-fed mice and MCD diet-fed mice, respectively. CYP3A11 mRNA levels were also similarly increased in TCPOBOP-treated animals. Administration of the CAR inverse agonist, androstanol[10,25], did not alter expression of these gene products (Figure 1A and B). Androstanol was, therefore, used as a negative control for CAR target genes in this model, as it blocks basal activity of CAR[26]. Unexpectedly, CYP2B10 and CYP3A11 mRNA levels remained elevated for 2 wk after initial TCPOBOP administration, mRNA expression of these target enzymes began to decrease by week three (down to 7-fold elevation) and further decreased by week four (down to 6-fold elevation) (data not shown). This data illustrates that TCPOBOP is a potent CAR agonist in the fatty liver and its effects are long-lasting after initial treatment.

Effects of TCPOBOP on hepatic and serum lipid content in MCD diet-fed mice

Animals treated with TCPOBOP and fed the MCD diet for 2 wk had 25% less hepatic steatosis than animals fed the MCD diet alone (Figure 2A). Interestingly, MCD diet-fed animals treated with androstanol had 15% more hepatic steatosis per surface area than untreated MCD diet-fed littermates. Of note, the fat pattern observed in the androstanol-MCD group was homogenously macrovesicular, where in untreated MCD diet-fed animals, the steatosis pattern was heterogeneous with both

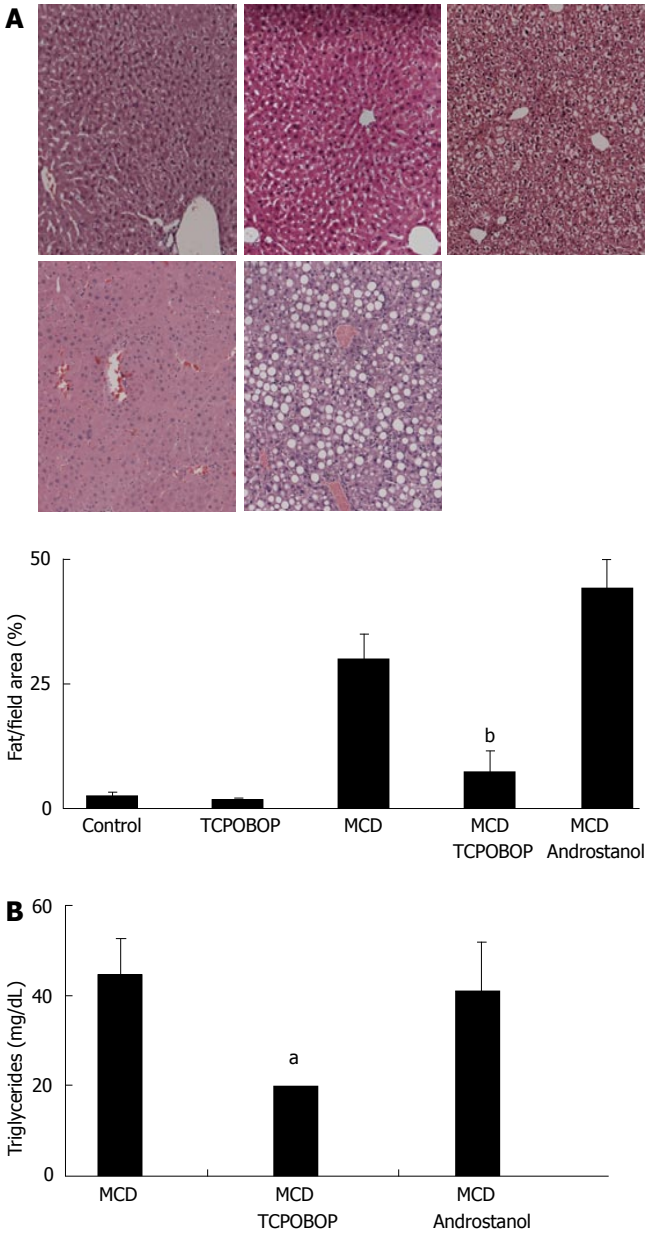

Figure 2 Hepatic and serum fat content reduced by TCPOBOP treatment in the MCD diet-fed mice. (**A**) Top panel, from left to right: Fixed liver specimens from vehicle-treated chow-fed mice, TCPOBOP-treated (3 mg/kg ip for 3 d) chow-fed mice, vehicle-treated (corn oil) MCD diet-fed diet mice for 2 wk, TCPOBOP-treated MCD diet-fed mice for 2 wk; CAR inhibitor, androstanol-treated MCD diet-fed mice for 2 wk were stained by conventional H&E. Lower panel: Quantitation of hepatic fat content by Oil red O staining. Note the marked reduction of hepatic fat in the TCPOBOP-treated MCD diet-fed mice (^{b}P = 0.001); (**B**) Serum triglyceride levels from mice fed the MCD diet for 2 wk with either TCPOBOP or androstanol treatment. Treatment with TCPOBOP reduced serum triglyceride levels by half (^{a}P = 0.03).

microvesicular and macrovesicular steatosis. Administration of TCPOBOP to the MCD diet-fed animals also markedly lowered serum triglyceride levels by 2-fold (Figure 2B). As expected, treatment with androstanol did not alter serum triglyceride levels. Collectively, these data demonstrate that TCPOBOP treatment decreased hepatic fat accumulation and improved serum triglyceride levels.

Modulation of hepatic expression of genes involved in fatty acid oxidation by TCPOBOP

The reduction in hepatic steatosis was consistent with stimulation of fatty acid oxidation by TCPOBOP. Therefore, we evaluated the effect of TCPOBOP on expression of several target genes involved in fatty acid oxidation (Table 2). In the liver, microsomal, mitochondrial and peroxisomal fatty acid oxidation systems are regulated by PPAR-α. PPAR-α mRNA expression levels were not altered by treatment with TCPOBOP in the MCD diet-fed animal (data not shown). However, CYP4A10 and CYP4A14, target enzymes for PPAR-α involved in the microsomal ω-oxidation system, were altered by TCPOBOP administration (Table 2). CYP4a10 mRNA expression was increased 4-fold ($P < 0.001$), while, CYP4A14 mRNA expression was actually decreased by TCPOBOP treatment ($P < 0.01$). L-PBE and peroxisomal 3-ketoacyl-CoA thiolase (ketoacyl thiolase), genes involved in peroxisomal β-oxidation were also significantly modified by TCPOBOP. mRNA expression was increased 6-fold for L-PBE, ($P < 0.001$) and 2-fold for ketoacyl thiolase ($P < 0.05$), when compared to MCD diet-fed untreated animals (Table 2). Two key genes involved in mitochondrial β-oxidation, carnitine palmitoyltransferase (CPT1) and mitochondrial trifunctional protein (MTP), showed no significant difference between TCPOBOP-treated and -untreated MCD diet-fed mice. These data demonstrated a PPAR-α-like effect on microsomal ω-oxidation and peroxisomal β-oxidation by TCPOBOP, which may confer protection against steatosis in the MCD diet-fed animal.

Table 2 Effect of TCPOBOP on genes involved in fatty acid oxidation

Type of fatty acid oxidation	Gene	MCD	MCD-TCPOBOP[a]	Fold-change	*P* value
ω-oxidation	CYP4A10	1.40 ± 1.69	6.07 ± 1.73	4.3	0.001
(ER)	CYP4A14	0.81 ± 0.04	0.07 ± 0.02	-11.6	0.008
β-oxidation	L-PBE	0.07 ± 0.01	0.39 ± 0.01	5.6	0.0004
(peroxisome)	Ketoacyl thiolase	0.12 ± 0.03	0.28 ± 0.06	2.3	0.04
	ACO	0.25 ± 0.14	0.32 ± 0.08	1.3	NS
	Urate oxidase	0.29 ± 0.02	0.16 ± 0.02	-1.8	NS
β-oxidation	CPT1	0.79 ± 0.21	0.77 ± 0.19	-1.0	NS
(mitochondria)	MTP-α	0.60 ± 0.30	0.88 ± 0.71	1.5	NS
	MTP-β	0.50 ± 0.40	0.59 ± 0.07	1.2	NS

Values are expressed as mRNA expression/18S ratio ± SD. [a]Animals fed methionine choline-deficient (MCD) diet for 2 wk and pre-treated with the CAR agonist, TCPOBOP (3 mg/kg ip for 3 d at starting of diet). NS: Not significant; ER: Endoplasmic reticulum; L-PBE: L-peroxisomal bifunctional enzyme; ACO; Acetyl-CoA oxidase; Ketoacyl thiolase: Peroxisomal 3-ketoacyl-CoA thiolase; CPT1: Carnitine palmitoyltransferase; MTP: Mitochondrial trifunctional protein subunits alpha and beta.

Alteration of apoptosis and inflammation in the MCD diet-fed animals by TCPOBOP treatment

Although TCPOBOP reduced hepatic steatosis, this reduction may or may not be sufficient to attenuate liver inflammation. Therefore, we assessed the effect of TCPOBOP on hepatocyte apoptosis and its effect on inflammation by quantitating macrophage/Kupffer cell numbers. Apoptosis was assessed by quantitating TUNEL- and caspase 3/7-positive cells. TUNEL- and caspase 3/7-positive cells were reduced in TCPOBOP-treated MCD diet-fed mice; this reduction of apoptosis was not observed with androstanol treatment (Figure 3A and B). Hepatic inflammation was assessed by

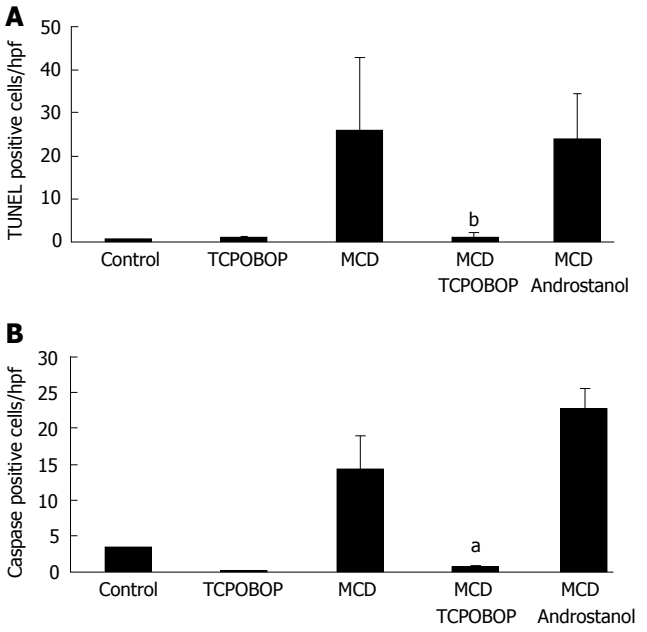

Figure 3 Hepatocyte apoptosis is attenuated by TCPOBOP in the MCD-diet fed animal. (**A** and **B**) Fixed liver specimens were analyzed by TUNEL and immunofluorescence for active caspase 3/7 to identify apoptotic liver cells. The percent/field area of TUNEL- (^bP = 0.01) and active caspase 3/7-positive cells (^aP = 0.03) were significantly higher in vehicle-treated MCD diet-fed mice than TCPOBOP-treated (3 mg/kg ip for 3d) MCD diet-fed mice (n = 5 in each group).

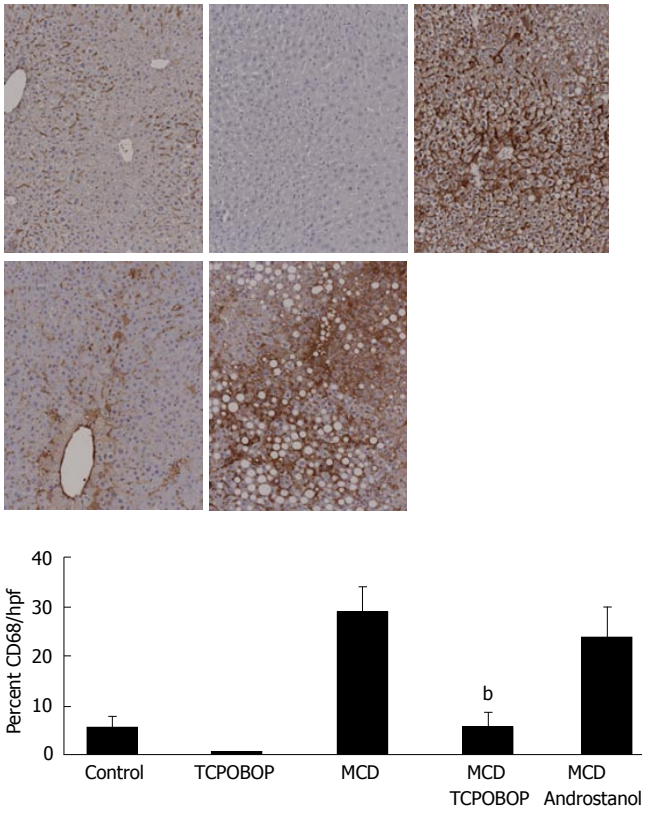

Figure 4 Hepatic inflammation attenuated by TCPOBOP treatment in the MCD diet-fed mice. Top panel: Representative photomicrographs of immunohistochemistry for CD68, a marker of Kupffer cells; Lower panel: The percentage of CD68-positive areas in the liver sections was quantitated using digital image analysis. Immunoreactivity for CD68 was significantly reduced in TCPOBOP-treated MCD diet-fed mice as compared with vehicle- and androstanol-treated mice (^bP = 0.002) (n = 5 in each group).

immunohistochemical staining for CD68, a marker for macrophage/Kupffer cells[27] (Figure 4). CD68-positive cells were less abundant in the MCD diet-fed mice treated with TCPOBOP as compared with the untreated mice on MCD diet; androstanol treatment did not reduce the number of CD68 immunoreactive cells. Consistent with these findings, leukotriene B4 (LTB4), a potent chemotactic agent that initiates, sustains and amplifies the inflammatory response, was also reduced in TCPOBOP-treated MCD diet-fed animals (55.6 ± 2.8 pg/mL) as compared to untreated MCD diet-fed mice (266.9 ± 12.1 pg/mL) (P = 0.001). These results suggest that TCPOBOP pre-treatment abrogates apoptosis and inflammation induced by the MCD diet.

DISCUSSION

The principle findings of this study relate to the effect of CAR modulation of steatohepatitis. The observations suggest TCPOBOP stimulation of CAR in the MCD diet-fed mice model of NASH: (1) reduces hepatic steatosis; (2) increases expression of genes involved in microsomal ω-oxidation and peroxisomal β-oxidation pathways; (3) and reduces hepatic inflammation.

We utilized the MCD animal model of steatosis for this study. The MCD diet stimulates steatosis by inhibition of fatty acid oxidation[15]. This animal model of hepatic steatosis is unique in that mice fed the MCD diet develop inflammation, thereby mimicking human NASH. Recently, peroxisome proliferator-activated receptor α (PPAR-α) agonists have been shown to be useful in ameliorating steatohepatitis induced by the MCD diet in mice by increasing genes involved in fatty acid oxidation[28,29]. PPAR-α is a transcription factor belonging to the nuclear receptor superfamily (NRSF) that increases hepatic uptake and breakdown of fatty acids by up-regulating genes involved in peroxisomal and mitochondrial β-oxidation and microsomal ω-oxidation[5]. In our current study, TCPOBOP administration demonstrated a PPAR-α-like effect on fatty acid microsomal ω-oxidation and peroxisomal β-oxidation enzymes, by increasing expression of CYP4A10, L-PBE and 3-ketoacyl-CoA thiolase. Our observations are consistent with findings from other studies in which protection from hepatic steatosis was afforded by changes in fatty acid microsomal ω-oxidation and peroxisomal β-oxidation without alteration of genes involved in the mitochondrial β-oxidation pathway[30]. These results indicate that upregulation of peroxisomal and microsomal enzymes was primarily responsible for the CAR-dependent reduction in hepatic steatosis.

Our data indicated that CAR activation by TCPOBOP had a PPAR-α-like effect on enhancing expression of genes involved in fatty acid oxidation. How CAR activation mimics PPAR-α activation is unclear, but suggests a potential interaction between the two nuclear receptors. CAR binds to its cognate DNA motif as a heterodimer with the retinoid X receptor (RXR), which serves as a common heterodimerization partner for other nuclear receptors in the superfamily, such as PPAR-α, pregnane X receptor (PXR), liver X receptor (LXR), and farnesol

X receptor (FXR)[31]. Although evidence exists for cross-talk between nuclear receptors of this superfamily[32,33], it is unknown if there is a direct link between CAR and PPAR-α activation. CAR could potentially bind to PPAR-α/RXR heterodimer, as LXR has been shown to bind directly to the PPAR-α/RXR complex[34]. This may afford CAR's ability to enhance expression of PPAR-α target genes involved in fatty acid oxidation. CAR has been shown to increase expression of gene targets otherwise thought to be regulated by other nuclear receptors. Recent experiments have illustrated CAR's ability to activate CYP3A, normally thought as the target of PXR[35]. Further studies are necessary to define the nature of the potential cross-talk between CAR and PPAR-α.

Apoptosis of markedly steatotic hepatocytes may incite the inflammatory response in NASH[36]. Apoptotic markers were reduced in TCPOBOP-treated MCD diet-fed animals in the present study. Indeed, previous work by us[13] has demonstrated CAR to have anti-apoptotic properties. In these studies, CAR activation by TCPOBOP depleted hepatocytes of the pro-apoptotic proteins Bak and Bax and increased expression of the potent anti-apoptotic protein Mcl-1 by directly promoting Mcl-1 transcription. These CAR-dependent processes rendered the liver resistant to death receptor-induced liver injury by Fas. The steatotic liver is highly susceptible to Fas-mediated apoptosis[37]. Perhaps, the CAR-induced resistance to Fas or other death ligands, such as tumor necrosis factor-alpha (TNF-α), accounts for its cytoprotective effects on the MCD diet-fed mouse. Finally, we noted that CAR can modify bile acid metabolism, and the role of bile acids in lipotoxicity remains unexplored[38].

The accumulation of fat in hepatocytes (steatosis) and the onset of steatohepatitis may reflect successive stages in fatty liver disease. The "two-hit" hypothesis postulates that the steatotic liver is susceptible to secondary insults including vulnerability to reactive oxygen species, tumor necrosis factor-α and other cytokines, which ultimately culminates in a sustained inflammatory response[39]. TCPOBOP treatment of MCD diet-fed mice resulted in a marked reduction in steatosis as well as inflammation. CAR-stimulated MCD diet-fed animals had decreased hepatic macrophage/Kupffer cell numbers and less circulating serum leukotriene B4 (LTB4). LTB4 is a potent chemotactic agent that initiates, sustains and amplifies the inflammatory response. Catabolism of LTB4 occurs in hepatocytes, as the liver is the principle organ for clearance of LTB4 from circulating blood[40]. In the liver, degradation of the fatty acid-like derivative LTB4 is modulated by PPAR-α regulation of microsomal ω-oxidation and peroxisomal β-oxidation pathways. Upregulation of PPAR-α-regulated genes involved in fatty acid oxidation by CAR activation may have attributed to a reduction in the inflammatory response, in part by enhancing LTB4 hepatic clearance.

In summary, our findings suggest that CAR activation is sufficient to attenuate hepatic steatosis, apoptosis and inflammation in the MCD diet-fed mice. The CAR-dependent PPAR-α-like stimulation of genes involved in microsomal ω-oxidation and peroxisomal β-oxidation pathways may, in part, be responsible for the hepatic cytoprotective effects observed in this model. These data also implicate a mechanistic link between fatty acid oxidation, hepatocyte apoptosis and Kupffer cell infiltration or inflammation. These preclinical studies suggest the employment of CAR agonists in the treatment of fatty liver diseases merits further attention.

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COMMENTS

Background

Nonalcoholic steatohepatitis (NASH) is a progressive spectrum of fatty liver disease for which there are limited therapeutic options. Apoptosis or programmed cellular death is a prominent histopathologic feature of NASH and correlates with disease severity. Recently, the constitutive androstane receptor (CAR), a nuclear receptor, has been reported to promote hepatic cytoprotection against apoptosis. Therefore, we hypothesized that CAR may ameliorate apoptosis induced by NASH.

Research frontiers

The incidence of nonalcoholic steatohepatitis is increasing with the rise in obesity over the last decade. New therapeutic regimens to slow down the process of fibrosis and cirrhosis in these patients will be important.

Innovations and breakthroughs

There is a prominent role for CAR cytoprotection against Fas-mediated hepatocyte injury *via* a mechanism involving upregulation of Mcl-1 (anti-apoptotic proteins) and, likely, downregulation of Bax and Bak (pro-apoptotic proteins).

Applications

At this time the use of CAR agonists to reduce the liver injury caused by NASH-induced hepatocyte apoptosis is still in its early phase of development. We need more data to fully evaluate its role in NASH.

Terminology

CAR is highly expressed in the liver and the small intestine, two key tissues expressing xenobiotic metabolizing enzymes, and mediates the induction of their expression by the widely used antiepileptic drug, phenobarbital (PB) and the potent synthetic inducer 1,4-bis-[2-(3,5,-dichloropyridyloxy)] benzene (TCPOBOP). TCPOBOP is an agonist ligand for CAR. PB induces its nuclear translocation, which results in increased expression of CAR target genes. The nuclear receptor, peroxisome proliferator-activated receptor alpha (PPAR-α), mediates many, if not all, of the adaptive consequences of peroxisome proliferator exposure in the liver, including alteration in lipid metabolism genes, hepatomegaly, and increases in liver tumors. Apoptosis is executed by caspases, a family of proteases that sequentially disassemble a cell. The pathways leading to caspase activation are dependent on the cytotoxic stimulus. Cytotoxic stress activates caspases by initiating signaling pathways that converge on the Bcl-2 family of proteins. After apoptotic stimulation, changes in the balance between pro- and anti-apoptotic members of this family lead to alteration of mitochondrial pore structure or integrity and permeabilization and release of proteins that promote cell death.

Peer review

This paper describes a series of experiments investigating the modulation of the CAR of steatotohepatitis in experimental rat model of NASH. The paper is well written and the methodology seems adequate. There are some issues that have to be addressed.

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RAPID COMMUNICATION

Viral blips during long-term treatment with standard or double dose lamivudine in HBe antigen negative chronic hepatitis B

Gianfranca Stornaiuolo, Maria Stanzione, Giuseppina Brancaccio, Gianluca Cuomo, Vincenza Precone, Sebastiano Di Biase, Francesca M Felaco, Felice Piccinino, Giovanni B Gaeta

Gianfranca Stornaiuolo, Giuseppina Brancaccio, Gianluca Cuomo, Vincenza Precone, Giovanni B Gaeta, Viral Hepatitis Unit, Department of Infectious Diseases, Second University, Naples, Italy
Maria Stanzione, Francesca M Felaco, Felice Piccinino, Infectious Disease Unit, Department of Infectious Diseases, Second University, Naples, Italy
Sebastiano Di Biase, Merigen, Laboratory of Molecular Biology, Naples, Italy
Correspondence to: Giovanni B Gaeta, Professor, Viral Hepatitis Unit, Department of Infectious Diseases, Second University of Naples, Via Cotugno 1, c/o Osp. Gesù e Maria, Naples 80135, Italy. giovannib.gaeta@unina2.it
Telephone: +39-81-5666208
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Abstract

AIM: To evaluate safety and effect on hepatitis B virus (HBV) suppression of a long-term treatment with lamivudine (LAM) at standard (100 mg/d) or double (200 mg/d) dose in chronic hepatitis B.

METHODS: This was a case study with matched controls (1:3) in patients with chronic hepatitis B with anti-HBe antibodies.

RESULTS: Twelve patients received LAM 200 mg/d and 35 LAM 100 mg/d, for a median of 28 mo. A primary response (PR; i.e., negative HBV-DNA with Amplicor assay) was achieved in 100% of LAM-200 patients and 83% of LAM-100 patients. A virological breakthrough occurred in 16.7 and 24.7%, respectively, of the PR-patients, with the appearance of typical LAM resistance mutations in all but one patient. Viremia blips (i.e., transient HBV-DNA below 80 IU/mL in patients who tested negative at Amplicor assay) were detected using a real time polymerase chain reaction (PCR) and occurred in seven out of nine patients with subsequent BT and in four out of 32 patients with end-of-study response (77.7% *vs* 12.5%; $P = 0.001$) at chi-square test). At the end of the study, 51.4% of LAM-100 patients and 83.3% of LAM-200 patients had remained stably HBV-DNA negative. Double-dose LAM was well tolerated.

CONCLUSION: Long-term treatment of anti-HBe positive chronic hepatitis B with double dose lamivudine causes a more profound and stable viral suppression as compared to conventional treatment.

Key words: Lamivudine; Hepatitis B; Chronic; Hepatitis; Polymerase chain reaction

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INTRODUCTION

Lamivudine (LAM) treatment has changed the therapeutic approach to chronic hepatitis B (CHB), since it has a potent antiviral effect and an excellent tolerability profile[1-4]. In chronic hepatitis B with anti-HBe antibodies, lamivudine must be administered indefinitely, since drug discontinuation causes an immediate rebound in the vast majority of patients[5,6]. Unfortunately, the drug has a low genetic barrier which causes the emergence of resistant mutants and disease resurgence at a rate of approximately 15% a year[7-10]. In addition, a percentage of patients ranging from 11% to 32% are primary non-responders to treatment[11-14], which limits further the efficacy of lamivudine in this setting.

At present, about 80% of patients with CHB in the Mediterranean area lack HBeAg in serum and present anti-HBe antibodies with the presence of HBV-DNA in serum[15]. This percentage is expanding in other parts of the world[16], due to the control measures against HBV and the subsequent decline in the proportion of hepatitis B virus (HBV) infections in young patients.

Lamivudine is registered for the treatment of chronic hepatitis B at a standard daily dose of 100 mg. This dosage derives from studies which compared the efficacy of 25, 100 and 300 mg/d given for 12-24 wk and found that viral suppression was similar at 100 and 300 mg[17,18]. As a consequence, 100 mg was adopted as the standard of therapy and the potential of higher doses in improving long-term outcomes has never been investigated.

In this pilot study we investigated the feasibility and the effects on viral suppression of a long-term treatment with a daily dose of 200 mg lamivudine in patients with HBeAg-negative chronic hepatitis B compared with matched patients treated with the standard dose.

MATERIALS AND METHODS

The study enrolled consecutive patients with HBeAg-negative, anti-HBe positive CHB between June 1999 and December 2002. The patients had to be negative for anti-HDV, anti-HCV and anti-HIV antibodies and present HBV-DNA in serum at levels $> 2 \times 10^4$ IU/mL. Basal evaluation comprised routine liver function tests, abdominal ultrasound (US) and liver biopsy. Histological specimens were evaluated using the Ishak's scores for necroinflammation and fibrosis[19]; minimal requirements for a specimen to be evaluated were a length of at least 2 cm and the presence of $\geqslant$ 10 portal tracts.

Twelve consecutive patients received lamivudine at the daily dose of 200 mg (LAM 200; 100 mg b.i.d.). As a reference group, we selected three age matched patients for each case, with anti-HBe positive CHB treated in the same period with the standard dose of 100 mg according to an open label, long-term study. In both cases and controls, the therapy was continued indefinitely; the therapy was stopped in patients who did not clear HBV-DNA after 12 mo of treatment. The study was closed on June 30, 2005.

The patients were followed-up monthly, including medical examination and alanine aminotransaminase (ALT) determination; at each visit a serum sample was stored at -40℃ for HBV-DNA testing. An abdominal US was performed every six months. Lamivudine tablets were dispensed directly monthly.

HBV-DNA analysis

Routine quantitative testing for HBV-DNA in serum was performed using Amplicor HBV Monitor (Roche, Branchburg, NJ, USA; detection limit 80 IU/mL).

In addition, analysis of HBV-DNA in serum was performed by the use of a Real Time PCR (RT-PCR) and Sybr Green as a fluorescent intercalating agent of the double strand DNA. The reference standards used for the external calibration were the WHO hepatitis B virus DNA International Standards No. 97/746 and the working reagent HBV No. 98/780, both provided by the National Institute of Biological Standards and Controls. HBV-DNA was extracted from serum by a slightly modified guanidinium thiocyanate method. Briefly, 100 μL of serum or plasma were added to 400 μL of the extraction solution and 0.5 mL of 2-propanol were added to the tube. The mixture was centrifuged for 10 min, then supernatant was removed and pellets washed twice with 70% ethanol. Each sample was dissolved in 50 μL sterile water and 10 μL were used for PCR (sensitivity = 10 IU/mL).

Processed specimens were added to 40 μL of an amplification buffered (pH = 8.0) mixture containing 0.5 μmol/L each of the reverse and forward primers directed toward the highly conserved HBV pre-core/core region and Sybr Green (BMA Molecular Probe), 2.5 IU of Taq Gold (Applied Biosystem) and 0.5 IU of Uracil DNA Glycosylase (Amersham Life Science - USB). The PCR process was carried out with the following amplification cycles: 50℃ for 2 min; 94℃ for 10 min; two cycles, each of 1 min duration, at 94℃, 60℃ and 72℃; 38 cycles at 94℃, 60℃ and 72℃, of 20 s each. Real time detection was performed using a ABI PRISM 7000 Sequence Detection System (Applied Biosystem).

HBV polymerase mutant and HBV genotype assay

Polymerase mutants conferring LAM resistance were detected using a commercial assay (InnoLipa HBV-DR, Innogenetics, Gent, Belgium) in all patients who presented serum HBV-DNA reappearance while on therapy and in those who remained viremic from the beginning of the therapy. HBV genotypes were identified using the INNOLIPA HBV-Genotyping test (Innogenetics, Gent, Belgium).

Therapy outcome definitions

Virological response (VR) to treatment was a fall in HBV-DNA below 80 IU/mL with or without a biochemical response (BR). Patients who remained constantly serum HBV-DNA positive over a 12 mo therapy period were defined as primary non-responders (PNR). A virological breakthrough (VBT) was defined as an increase $\geqslant$ 1 log in serum HBV-DNA in a previous responder patient while continuing LAM therapy; a biochemical breakthrough (BBT) was an ALT elevation above 2 × in a virological BT. An ALT flare was an ALT elevation > 500 U/L. A viral blip was a transient HBV-DNA below 80 IU/mL detected by RT-PCR in patients who tested negative at Amplicor assay. Complete viral suppression was a stably undetectable HBV-DNA in serum using RT-PCR.

Safety

A clinical examination was performed monthly. At the same time, differential blood count and serum biochemistry were performed, including serum amylase, lipase and creatinine phosphokinase (CPK) determination. Any adverse event was registered.

Statistical analysis

The data were analyzed according to an intention to treat procedure. Patients who were lost at follow-up were considered as non-responders. Chi-square and Fisher's exact test were used to analyze categorical variables and Student's *t* test for continuous data. The data were analyzed using SPSS software, version 12. The study was approved by the local Ethics Committee.

RESULTS

Twelve patients received LAM 200 mg/d and 35 patients LAM 100 mg/d (one patient receiving 200 mg LAM was matched with two controls only). The basal characteristics of the patients are reported in Table 1. There was no difference between the groups relating to demographic data and ALT values, serum HBV-DNA values and liver histology. Overall, a liver biopsy taken within 18

Table 1 Baseline characteristics of the patients

	Lam-200	Lam-100	*P*
Number of patients	12	35	NS
Age (median; range)	44.5 (32-60)	44 (23-72)	NS
BMI median (range)	24.3 (23.2-28.7)	25.5 (20.9-34.3)	NS
Gender (M/F)	11/1	26/9	NS
Histology			NS
Minimal-Mild	1 (14%)	6 (22%)	
Moderate-Severe	3 (43%)	7 (26%)	
Cirrhosis	3 (43%)	14 (52%)	
ALT (x n.v.)			NS
< 3	4 (33.3%)	16 (50%)	
3-5	3 (25%)	5 (15.6%)	
> 5	5 (41.7%)	11 (34.4%)	
HBV-DNA IU/mL median (range)	1.06×10^6 (3.2×10^4-3.8×10^6)	6×10^5 (2.4×10^4-3.5×10^6)	NS
Months of therapy median (range)	28 (8-50)	28 (9-48)	NS

Table 2 Virological responses in the two treatment groups *n* (%)

	Lam-100 (*n* = 35)	Lam-200 (*n* = 12)
Primary responses	29 (82)	12 (100)
Breakthroughs	7 (24.6)	2 (16.6)
End of study responses (amplicor)	22 (62.8)	10 (83.3)
Stably undetectable HBV-DNA by RT-PCR	18 (51.4)[a]	10 (83.3)[a]

[a]*P* = 0.051; 51.4% *vs* 83.3%.

mo before therapy was available in 34 patients; 50% of whom had cirrhosis. In both groups median treatment duration was 28 mo. Based on Roche Monitor assay, a primary VR was observed in all the patients treated with 200 mg lamivudine and in 83% of those treated with the standard regimen (Table 2). In both groups, median HBV-DNA disappearance time was 3 mo and in no patients did HBV-DNA become undetectable at Monitor test later than the sixth months of therapy. Primary non-response was observed in six patients, all belonging to the 100 mg group. In PNRs LAM therapy was discontinued after 12 mo; during treatment, HBV-DNA levels remained above 2×10^4 IU/mL.

During treatment, a VBT was detected in nine patients, of whom two belonged to the 200 mg group (2/12; 16.6%) and seven to the 100 mg group (7/29; 24.1%). A BBT followed in all patients with VBT; in these patients, the ALT values were below 2 × the basal value in eight out of nine patients and in the range of a hepatitis flare in one patient (belonging to 100 mg group). At the end of the study, 63% of the patients in LAM-100 group and 83% in LAM-200 group were still VR (Table 2). None of the basal variables considered in Table 1 was associated with the outcome of the treatments.

In 11 patients who were constantly negative at the HBV-Monitor test, the RT-PCR revealed transient viremia blips below 80 IU/mL. This feature was recorded in seven out of the nine patients who subsequently presented a BT and in four out of the 32 patients with end of study response, the latter belonging to the 100 mg group (Table 3; $P = 0.001$).The negative predictive value and positive predictive value for BT were 0.93 and 0.63, respectively. Viremia blips occurred in 10 out of 29 (34.4%) patients who were initially responders to LAM 100 mg and in one out of 12 (8.4%) of those responding to LAM 200 mg. On the whole, the patients who showed absent or partial viral suppression (i.e., a PNR or a VBT or viral blips) were 17 out of 35 treated with LAM 100 mg and two out of 12 treated with LAM 200 mg ($P = 0.051$).

Lamivudine resistance mutations were researched in 13 cases, five of whom were PNRs and eight BTs (Table 4). None of the primary non responders had detectable mutations at baseline; however, all of them developed rtM204V/I mutant (none with concomitant rtL180M) while continuing LAM therapy, two with an ALT increase after the appearance of the mutation and one with a hepatitis-like flare. Among BT patients, seven presented rtM204V/I (four with concomitant rtL180M) and one patient presented an ALT flare with no detectable mutations. Mutations preceded the clinical BT by one to

Table 3 Viral blips observed during treatment. A viral blip is a transiently detectable viremia below 4×10^2 copies/mL in a responder patient

	Lam 100	Lam 200	Total
Primary responders (*n*)	29	12	41
Viral blips *n* (%)	10 (34.4)	1 (8.3)	11
Breakthroughs no.	7	2	9
Viral blips *n* (%)	6 (85.7)	1 (50)	7 (77.7)[a]
End of study responses (Amplicor)	22	10	32
Viral blips *n* (%)	4 (18.2)	0	4 (12.5)[a]

[a]Viral blips among patients with breakthroughs *vs* viral blips among patients with end of study response; 77.7% *vs* 12.5%; *P* = 0.001.

Table 4 Lamivudine resistance mutations and HBV genotypes in patients with a virological breakthrough or primary non-response

Patients	Genotype	Baseline	Mutation (mo)
Breakthrough			
[1]1	D	Wt	rtM204V (11)
[1]2	D	Wt	None (19)
[2]3	D	Wt	rtM204V (24)
[1]4	A	Wt	rtM204V rtL180M (28)
[1]5	D	Wt	rtM204V rtL180M (32)
[1]6	D	Wt	rtM204V rtL180M (19)
[1]7	D	Wt	rtM204V rtL180M (21)
[1]8	D	Wt	rtM204I (12)
Primary non-response			
[1]1	D	Wt	rtM204I (9)
[1]2	D	Wt	rtM204V (11)
[1]3	D	Wt	rtM204I (11)
[1]4	D	Wt	rtM204I (9)
[1]5	D	Wt	rtM204I (12)

Treatment group: [1]LAM:100; [2]LAM:200.

eight months. HBV genotypes were detected in 24 patients, all but one were genotype D.

Lamivudine was well tolerated; one patient belonging to the 100 mg group discontinued the treatment due to an increase in serum amylase to eight times the UNL. No serious adverse events were recorded in either treatment group.

DISCUSSION

This study was designed to check whether a long-term, double dose lamivudine treatment is feasible in patients with chronic hepatitis B and has the potential for a more profound and stable suppression of viral replication.

Basically, lamivudine pharmacokinetic is subjected to significant individual variability[20-22]. Plasma concentrations of lamivudine do not reflect its antiviral activity, since it depends on the 5-triphosphate anabolite of the drug, which is formed through an intracellular, saturable enzymatic process. Higher dosage of lamivudine produced higher intracellular lamivudine triphosphate concentrations. The intracellular half-life of active compound varies from 17 to 19 h in hepatic cell lines and from 10.5 to 15.5 h in peripheral blood mononuclear cells (PBMCs)[23,24] which suggested a dose interval of 12 h in HIV patients. In our study giving daily LAM 200 mg in two refracted doses may have enhanced intracellular active drug availability and even reached adequate anti-viral concentrations in extrahepatic sites of HBV replication.

In chronic hepatitis B, lamivudine doses ranging from 25 to 300 mg/d were used obtaining a steady viral inhibition at 100 mg[17,18] as measured by a hybridization assay. Looking at long term efficacy, Yuen *et al*[25] found no difference in the cumulative incidence of resistance mutations between patients treated with lamivudine 100 mg/d and those who received 25 mg/d for one to three years, though viral suppression was less effective at lamivudine 25 mg than at lamivudine 100 mg. This suggests that viral suppression may be sub-optimal even at the dose of 100 mg and is in keeping with our results at 100 and 200 mg.

In our study, the use of a sensitive PCR method highlighted that viral suppression was less efficient at 100 mg/d and this was the background for mutant selection and viral breakthroughs. Indeed, some patients classified as VR by Monitor assay had viremia blips below 80 IU/mL. This phenomenon was significantly associated with subsequent stable resurgence of viral replication and was mostly observed in patients receiving 100 mg lamivudine. At the end of the study a stable viral suppression was achieved in 83% of the patients receiving 200 mg LAM and in 51% of those treated at 100 mg/d.

There are some practical consequences from these observations, which are summarized in Figure 1. First of all, patients under treatment with lamivudine require strict follow-up with a high-sensitivity PCR assay, in order to detect, as soon as possible, even low level viremia. Secondly, an early change in therapeutic strategy is advisable for patients with viremia blips, by switching to adefovir or to entecavir[26-28], in order to prevent virological and biochemical BTs. Finally, patients with undetectable HBV-DNA and no viremia blips are candidates for continuing long-term lamivudine monotherapy, since the absence of viremia blips is predictive of long-term response.

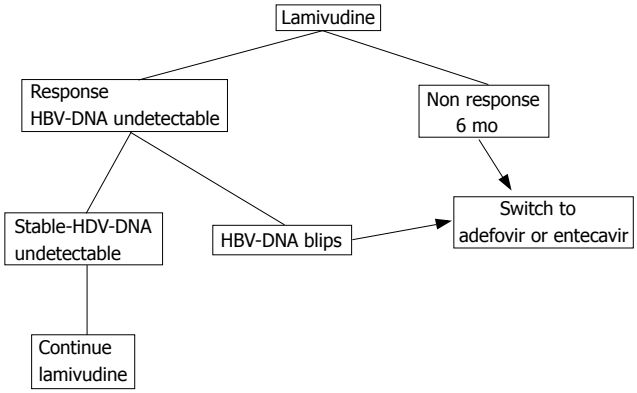

Figure 1 Flow-chart for patients under lamivudine treatment.

Genotype D of HBV was largely prevalent in our series and this raises the question as to whether the results are applicable to different HBV genotypes. There is evidence that response rates to Peg-interferon depend on HBV genotypes while less clear-cut results were obtained with anti-HBV analogs[1,29-31].

None of the primary non-response was associated with a pre-existing mutation, supporting the concept that the pharmacokinetic of the drug may play an important role in primary non-response and that higher drug doses may force this pharmacokinetic defect. Alternatively, PNR patients might harbour viral variants not detected by our method. Lamivudine resistance mutations were detected in all but one patient with virological BT.

In all primary non-responders continuing the treatment caused the appearance of resistance mutations, with further ALT elevation. From a practical point of view, patients who do not respond to a standard lamivudine dose should stop the treatment within the first six months (Figure 1), since a response after this period is unlikely. There is no data on early shifting to a higher lamivudine dose after an initial non-response to a standard dose.

In conclusion, our results indicate that the patients under lamivudine should be monitored using a high sensitivity PCR assay. An extended treatment with a double dose of lamivudine is feasible in chronic hepatitis B, and has the potential for a more pronounced viral suppression than a standard dose. Since lamivudine is a well-used, non-toxic and non-expensive anti-viral agent, these data should stimulate more powered studies aimed at optimizing treatment strategies.

COMMENTS

Background

Sustained viral suppression is the main goal of antiviral therapy in chronic hepatitis B. Lamivudine was the first antiviral drug approved for HBV treatment, at the standard daily dose of 100 mg. Although it was safe and potent, it caused the emergence of drug-resistant viral strains, at a rate of about 15% a year. At present, other antiviral drugs against HBV are available (adefovir, entecavir, telbivudine) that show a higher genetic barrier than lamivudine. Recent guidelines do not recommend the use of lamivudine as first line therapy in chronic hepatitis B.

Research frontiers

Thousands of patients with chronic hepatitis B are under lamivudine treatment.

Furthermore, lamivudine is the cheapest anti-HBV drug. After five years of continued treatment approximately 25% of the patients have an undetectable HBV-DNA. Can we improve the efficacy of lamivudine and predict the long-term response to treatment? There is a lack of studies with a higher than standard dosage of lamivudine in immunocompetent patients. This article examines the possibility of prolonged treatment with double daily dose of lamivudine (200 mg) and the predictive value of close monitoring of serum HBV-DNA using a highly sensitive real-time PCR.

Innovations and breakthroughs

Patients treated with lamivudine 200 mg/d achieved a more rapid primary response and a more profound and long-lasting viral suppression. In fact, the use of a sensitive PCR for HBV-DNA detection showed that transient low viremia levels ("viral blips") were more frequently detected in patients receiving lamivudine 100 mg and predicted a virological breakthrough and the emergence of lamivudine resistance mutations.

Applications

Patients under lamivudine treatment require a strict follow-up using a highly sensitive PCR. Real time PCR has a lower detection limit of 10 IU/mL and seems the best method for this purpose. Detecting even low level viremia during treatment may predict the emergence of resistance mutations. In this case, an early change in therapeutic strategy is advisable as indicated in Figure 1 of the paper. The paper suggests that therapy with double dose lamivudine is feasible and may achieve better results than the standard dose.

Terminology

Virological breakthrough is the increase of at least one log of the plasma viral concentration during anti-viral therapy. In most of the cases it is due to the emergence of viral resistant strains. Biochemical breakthrough is the increase of ALT value that follows the virological breakthrough. Genetic barrier may be defined as the probability of not reaching any resistant escape strains.

Peer review

The aim and content of the study were considered innovative. However, some limits come from the low number of patients enrolled in the 200 mg lamivudine group. As the authors stated, these results should encourage further trials.

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RAPID COMMUNICATION

Effect of sustained virological response on long-term clinical outcome in 113 patients with compensated hepatitis C-related cirrhosis treated by interferon alpha and ribavirin

Roland El Braks, Nathalie Ganne-Carrié, Hélène Fontaine, Jacques Paries, Véronique Grando-Lemaire, Michel Beaugrand, Stanislas Pol, Jean-Claude Trinchet

Roland El Braks, Nathalie Ganne-Carrié, Véronique Grando-Lemaire, Michel Beaugrand, Jean-Claude Trinchet, Department of Hepatology, Hopital Jean Verdier (AP-HP) and UPRES EA 3409 (University Paris 13), Bondy 93140, France
Hélène Fontaine, Stanislas Pol, Department of Hepatology, Hopital Cochin (AP-HP) and University Paris 5, Paris 75014, France
Jacques Paries, Department of Public Health, Jean Verdier Hospital (AP-HP) and UPRES EA 3409 (University Paris 13), Bondy 93140, France
Correspondence to: Nathalie Ganne-Carrié, Department of Hepatology, Hopital Jean Verdier, Bondy 93140, France. nathalie.ganne@jvr.aphp.fr
Telephone: +33-1-48026280 Fax: +33-1-48026202
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Abstract

AIM: To assess the long-term clinical benefit of sustained virological response (SVR) in patients with hepatitis C virus (HCV) cirrhosis treated by antiviral therapy using mostly ribavirin plus interferon either standard or pegylated.

METHODS: One hundred and thirteen patients with uncomplicated HCV biopsy-proven cirrhosis, treated by at least one course of antiviral treatment ⩾ 3 mo and followed ⩾ 30 mo were included. The occurrence of clinical events [hepatocellular carcinoma (HCC), decompensation and death] was compared in SVR and non SVR patients.

RESULTS: Seventy eight patients received bitherapy and 63 had repeat treatments. SVR was achieved in 37 patients (33%). During a mean follow-up of 7.7 years, clinical events occurred more frequently in non SVR than in SVR patients, with a significant difference for HCC (24/76 *vs* 1/37, $P = 0.01$). No SVR patient died while 20/76 non-SVR did ($P = 0.002$), mainly in relation to HCC (45%).

CONCLUSION: In patients with HCV-related cirrhosis, SVR is associated with a significant decrease in the incidence of HCC and mortality during a follow-up period of 7.7 years. This result is a strong argument to perform and repeat antiviral treatments in patients with compensated cirrhosis.

Key words: Hepatitis C; Cirrhosis; Interferon alpha

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INTRODUCTION

In patients with chronic liver diseases, the prognosis strongly depends on the extent of liver fibrosis, as life-threatening complications mainly occur in patients with cirrhosis. In chronic hepatitis C in particular, hepatocellular carcinoma (HCC) is observed only in case of cirrhosis or severe fibrosis (some of them presumably with under diagnosed cirrhosis due to sampling error of liver biopsy). In patients with compensated hepatitis C virus (HCV)-related cirrhosis, the annual incidence of HCC, decompensation and death reach around 3%, 4% and 3%, respectively[1-4] and the main cause of death is HCC[1].

Interferon-α was approved for use in chronic hepatitis C in 1991. During the past 15 years, subsequent improvements have included extension of therapy to 48 wk, the combination of interferon-α with ribavirin, and the use of pegylated interferon. These progresses have resulted in improvement in the sustained virological response (SVR) rate in patients included in randomized trials from less than 10% with the initially recommended 24-wk course of interferon-α monotherapy to as high as 50%-60% with the combination of peg interferon and ribavirin for 48 wk. In unselected patients, the percentage of SVR is expected to be lower particularly in patients with cirrhosis.

Most studies of antiviral therapy of HCV have been limited to 6 mo of follow-up after the end of treatment and have not included HCC, liver decompensation or death as end points. Therefore, studies of the effect of antiviral therapy on clinical outcome of HCV-cirrhosis have largely been retrospective analyses of therapeutic trials using interferon-α alone, focusing on patients with cirrhosis[4-11]. Only three randomized trials have assessed

this effect, giving conflicting results[12-15]. Recent meta-analysis suggested a slight, but significant preventive effect of standard interferon-α monotherapy on HCC occurrence in patients with HCV-cirrhosis, especially in those who achieve SVR, who intrinsically represent a small proportion of patients in these trials[16,17]. The influence of interferon-α on the incidence of decompensation or death in patients with HCV-cirrhosis was less studied and more controversial[4,8,10,12,14,18].

At present, bitherapy with standard then pegylated interferon plus ribavirin has created a new perspective for patients with HCV because of the higher rate of SVR reported[19,20]. The clinical long-term benefit of bitherapy with standard interferon-α has been recently assessed in Chinese patients with HCV-cirrhosis[21]. Such data are still not available in Western patients. Therefore, to assess the impact of achievement of a SVR on clinical long-term outcome of cirrhosis, we conducted a long-term, retrospective, bi-centre analysis of prospective collected data from a cohort of 113 French patients with histological proven HCV cirrhosis treated at least 3 mo by different regimens including ribavirin plus standard or pegylated interferon, and periodically followed and screened for HCC according to standardized criteria.

MATERIALS AND METHODS

Patients

We retrospectively selected all the consecutive patients followed-up in two French liver centres between 1989 and 2006 fulfilling the following criteria: (1) HCV-related cirrhosis defined by association of positive serum anti-HCV antibodies and RNA, with typical liver histology; (2) absence of complication before or at inclusion (Child-Pugh class A); (3) absence of HBV or HIV co infection (negative serum HBsAg and HIV antibodies); (4) daily alcohol consumption < 50 g; (5) absence of contra-indication to antiviral treatment, particularly platelet and polymorphonuclear counts ⩾ 80 000/mm^3 and 1500/mm^3, respectively; (6) at least a 3 mo course of antiviral treatment using standard or pegylated interferon with or without ribavirin, according to therapeutic advance over time and international guidelines; (7) a regular follow-up ⩾ 30 mo after the starting of the first treatment; (8) absence of HCC or even suspicious findings such as liver nodule or serum level of alpha-fetoprotein (AFP) above 50 ng/mL; (9) residence in France allowing regular follow up.

Assessment of Response to antiviral treatment

SVR was defined as undetectable serum HCV RNA 6-mo after discontinuation of the last treatment. Non responder patients to a first line treatment were retreated once or more each time it was possible with the same or a different regimen according to therapeutic advance over time. Serum HCV RNA was measured annually during follow-up and at the time of the last visit. According to final virological response, patients were separated into SVR and non SVR groups. Patients not fulfilling SVR criteria, including all patients who relapsed after the achievement of the end of treatment response, were classified as non-SVR.

Follow-up

All patients were prospectively followed-up according to the same schedule in both centres. Complete physical examination, standard biochemical tests, serum AFP determination and abdominal ultrasonography (US) were repeated every 6 mo, whatever the virological response. Baseline then annual endoscopy of the upper gastrointestinal tract allows assessment of the presence of gastrooesophageal varices. In case of significant endoscopic portal hypertension, prophylactic treatment (propranolol and/or endoscopic treatment) was started. The length of the study was calculated from the starting date of antiviral therapy and ended at death or at the last follow-up visit.

Endpoints

When a focal liver lesion or increased AFP levels were detected, tomodensitometry and, whenever possible, fine needle guided liver biopsy were performed. Diagnostic criteria for HCC were: (1) histological and (2) clinical, in patients with AFP value greater than 400 ng/mL and evidence of focal liver lesion at imaging techniques. After 2002, the HCC diagnosis was based on the guidelines of the European Association for the Study of the Liver[22].

Liver-related complications (ascites, upper gastro-intestinal bleeding, and hepatic encephalopathy) were considered an endpoint in all patients with or without the occurrence of HCC. In the subjects who developed HCC, liver complications were recorded only when they occurred before tumour development. Ascites was diagnosed by clinical examination and/or US detection. Porto-systemic encephalopathy was defined by clinical parameters. The source of gastro oesophageal bleeding was confirmed by endoscopy whenever possible.

Dates and causes of deaths were recorded. Liver transplantation was considered as liver-related death endpoint. Reference date was February 2006.

Statistical analysis

Continuous variables are reported as mean and standard deviation, and categorical variables as absolute and relative frequencies. The Mann-Whitney rank-sum test (M-W) and the Kruskal-Wallis nonparametric analysis of variance (K-W) were applied to compare the means. The associations between Non-SVR or SVR status and events in 2 × 2 cross tabulations were tested using Fisher's exact test. In case of larger cross tabulations, we tested the correlation by computing Pearson's Chi-square, or by computing either the exact probability value, or the Monte Carlo estimate of the exact probability value. Cumulative incidence curves of liver-related complications, HCC and mortality according to response to interferon treatment were plotted using the Kaplan-Meier method. The differences between groups were assessed using log-rank tests. Data was censored when individuals died, received a liver transplantation or were lost during follow-up. The variables which proved to be significant at univariate analysis were tested by the multivariate Cox proportional hazards regression model to assess their independent effect on the development of events during the follow-up. The

Table 1 Baseline characteristics of patients, as a whole and according to final virological response

Characteristics	All $n = 113$	SVR $n = 37$	Non SVR $n = 76$	P^1
Male gender (%)	69 (61.1%)	31 (83.8%)	38 (50.0%)	0.0005
Age (mean, yr)	54.1 ± 11.2	50.6 ± 11.1	55.8 ± 10.9	0.02
Alcohol (g/d)				0.4
0	71 (62.8%)	21 (56.8%)	50 (65.8%)	
< 20	20 (17.7%)	9 (24.3%)	11 (14.5%)	
⩾ 20	22 (19.5%)	7 (18.9%)	15 (19.7%)	
HCV genotype[2]				0.0001
1	58 (61.1%)	11 (35.5%)	47 (73.4%)	
2	10 (10.5%)	7 (22.6%)	3 (4.7%)	
3	15 (15.8%)	8 (25.8%)	7 (10.9%)	
4	8 (8.4%)	1 (3.2%)	7 (10.9%)	
5	2 (2.1%)	2 (6.5%)	0 (0.0%)	
6	2 (2.1%)	2 (6.5%)	0 (0.0%)	
Body mass index (kg/m^2)	25.9 ± 4.0	24.6 ± 3.8	26.5 ± 4.0	0.015
Platelets (10^3/mm^3)	149.4 ± 56.2	152.9 ± 50.1	147.9 ± 59.2	0.4
Serum albumin (g/L)	43.0 ± 5.0	45.4 ± 6.1	41.8 ± 4.0	0.001
Bilirubin (μmol/L)	14.6 ± 9.6	14.3 ± 7.5	14.8 ± 10.5	0.6
Prothrombin activity (%)	83.7 ± 13.3	84.5 ± 12.6	83.4 ± 13.8	0.7
AFP (ng/mL)	14.4 ± 25.2	8.7 ± 12.9	17.3 ± 29.2	0.008
AST (× ULN)	2.9 ± 2.0	2.4 ± 1.4	3.1 ± 2.3	0.2
ALT (× ULN)	3.1 ± 1.9	3.1 ± 1.9	3.1 ± 2.0	0.7

ULN: Upper limit of normal range; SVR: Sustained virological response. [1]Between SVR and Non-SVR groups; [2]Not determinate in 18 patients (15.9%).

Table 2 Duration of follow-up and characteristics of treatment according to final virological response

	All $n = 113$	SVR $n = 37$	Non SVR $n = 76$	P^1
Follow-up from the first liver biopsy (mean ± SD, yr)	8.2 ± 3.1	8.2 ± 2.7	8.2 ± 3.3	0.7
Follow-up from the beginning of first treatment (mean ± SD, yr)	7.7 ± 3.0	7.7 ± 2.6	7.6 ± 3.1	0.6
Number of treatment courses				0.25
1	50 (44.2%)	19 (51.4%)	31 (40.8%)	
2	42 (37.2%)	15 (40.5%)	27 (35.5%)	
3	19 (16.8%)	3 (8.1%)	16 (21.1%)	
4	2 (1.8%)	0 (0.0%)	2 (2.6%)	
Type of treatment				1.00
α-interferon monotherapy	35 (31.0%)	11 (29.7%)	24 (31.6%)	
Bitherapy	78 (69.0%)	26 (70.3%)	52 (68.4%)	
Type of bitherapy				< 0.0001
α-interferon + ribavirin	40 (51.3%)	22 (84.6%)	18 (34.6%)	
Peg-interferon + ribavirin	38 (48.7%)	4 (15.4%)	34 (65.4%)	
Total duration of treatment (mo)	15.3 ± 9.05	14.3 ± 6.4	15.8 ± 10.1	0.4

SVR: Sustained virological response. [1]*P* values were obtained between SVR and non SVR groups.

results were expressed as hazard ratio (HR) and their 95% confidence interval (CI). Data handling and analysis were performed with the Statistical Package for Social Sciences (SPSS 13.0; SPSS Inc., Chicago, IL) and STATXACT. All tests were 2-sided and $P < 0.05$ was considered to be statistically significant.

RESULTS

Patients and treatment regimens

One hundred thirteen patients fulfilled inclusion criteria (mean age: 54 ± 11 years, males: 61%, HCV genotype 1: 51%) (Table 1). As a whole, the mean (± SD) cumulated duration of treatment was 15.3 ± 9 (range: 3-42, median 12) mo. Treatment courses were performed 1, 2, 3 and 4 times in 50 (44.2%), 42 (37.2%), 19 (16.8%) and 2 (1.8%) patients respectively (Table 2). Interferon-ribavirin bitherapy was administered in 78 patients (69%), using pegylated interferon in 38 cases. SVR was obtained in 37 patients (33%). Nineteen out of 113 treated patients (16.8%) became SVR after the first treatment, 15/113 (13.3%) after the second, 3/113 (2.7%) after the third and 0/113 (0%) after the fourth treatment. Among the 37 SVR patients, 11 (29.7%) received only a monotherapy versus 24 (31.6%) in the 76 non SVR group ($P = 1.00$). Cumulated duration of antiviral treatment (14 mo ± 6 mo *vs* 16 mo ± 10 mo) did not significantly differ between SVR and non SVR patients ($P = 0.99$). There was no significant difference between the 2 groups regarding the number and the patterns of treatment (Table 2). Furthermore, there was no heterogeneity among the 2 centres in terms of duration and patterns of treatment.

The main patients' baseline characteristics according to final response are reported in Table 1. Compared to non SVR, SVR patients were more often males (84% *vs* 50%, $P = 0.0005$), younger (51 ± 11 *vs* 56 ± 11 years, $P = 0.02$), leaner (25 *vs* 26.5 kg/m^2, $P = 0.015$), less frequently infected with HCV genotype 1 (35.5% *vs* 73.4%, $P = 0.0001$), and had higher serum albumin levels (45 *vs* 42 g/L, $P = 0.001$) and lower AFP serum level (8.7 *vs* 17.3 ng/ mL, $P = 0.008$).

Follow-up and clinical events occurrence

The mean duration of follow-up was 7.7 ± 3.0 years from the beginning of the first treatment in the whole population. SVR and non SVR patients did not differ in term of follow-up (7.7 ± 2.6 *vs* 7.6 ± 3.1 years, $P = 0.6$) (Table 2).

During the follow-up, at least one severe clinical event (HCC or ascites or hepatic encephalopathy or gastrointestinal bleeding or death) occurred in 37 patients with a significant higher frequency in non-SVR (44.7% versus 8.1%, Hazard Ratio 6.3 (95% Confidence Interval 1.9-20.4), $P = 0.002$). Ascites, hepatic encephalopathy or gastrointestinal bleeding occurred in 11/76 non-SVR and in 3/37 SVR (Figure 1); HCC occurred in 24/76 non-SVR and in 1/37 SVR (Figure 2). The difference between the two groups was significant only for HCC [$P = 0.01$, Hazard Ratio 13.6 (95% Confidence Interval 1.8-100.5)], while there was no significant difference for the occurrence of other clinical events [ascites, hepatic encephalopathy or gastrointestinal bleeding: $P = 0.44$, Hazard Ratio 1.65 (95% Confidence Interval 0.46-5.9)] (Table 3). The diagnosis of HCC was assessed by histology in 9 patients (before June 2002) and according to guidelines of European Association for the Study of the Liver[22] in 16 patients (after July 2002). The median number of nodules was 1.5 (range, 1-8) and the median size 30 (range, 10-65) mm. The median times from the beginning of the first treatment until complications were not different in SVR and non-SVR: 6.99 years in SVR and 6.36 years in non-SVR for the occurrence of HCC ($P = 0.150$); 6.91 years in SVR and

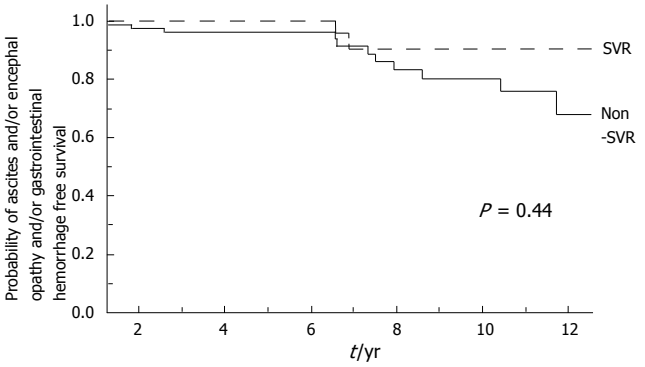

Figure 1 Incidence of ascites or encephalopathy or gastrointestinal haemorrhage from the beginning of the first treatment according to the response to antiviral treatment (SVR: Sustained virological response in dotted line; non-SVR in full line; Log-Rank, $P = 0.44$).

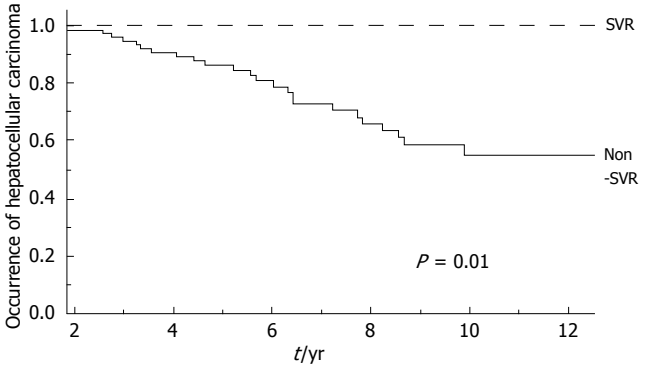

Figure 2 Incidence of hepatocellular carcinoma from the beginning of the first treatment according to response to antiviral treatment. SVR: Sustained virological response in dotted line; Non-SVR in full line; Log-Rank, $P = 0.01$.

6.78 years in non-SVR for the onset of decompensation or upper GI bleeding ($P = 0.531$). For the 37 patients who achieved a SVR, the median time from SVR (i.e., 6 mo after the end of the successful antiviral treatment) to the occurrence of complication was 4.79 years for HCC and 4.66 for decompensation. Death rate was significantly higher in case of non-SVR patients (20/76 *vs* 0/37; $P = 0.002$, Figure 3). Deaths were mainly related to liver disease and HCC was causative of 45% of the deaths. There was no heterogeneity among the 2 centres in terms of patterns of the duration of follow-up and of the number of events observed.

The multivariate analysis (Cox model) found two independent predictive factors for clinical events: SVR [$P = 0.001$, HR 7.1 (95% CI 2.2; 23.2)] and duration of treatment [$P = 0.001$, HR 0.93 (95% CI 0.89; 0.97)]. In other words, the Hazard for HCC was 7% lower for each additional year of treatment.

There was no significant difference with age, gender, or viral genotype for HCC occurrence.

DISCUSSION

Our study demonstrates a significant difference in long-term outcome between initially uncomplicated HCV cirrhotic patients with and without SVR after antiviral treatment. Over a mean follow-up period of 7.7 years, there were no deaths and virtually no severe clinical events in patients with SVR. At the opposite, HCC and liver decompensation occurred in patients without SVR with incidence rates similar to those previously reported in untreated cirrhotic patients[23]. We should emphasize that HCC was carefully screened before treatment, which eliminates HCC of small size appearing a few months after antiviral treatment.

Several studies performed in patients with chronic hepatitis C have suggested that successful antiviral treatment could result in a dramatic decrease in severe clinical events and mortality, life expectancy of patients with SVR being close to general population[6,9,10,24,25]. However, those studies used only interferon-α. In the past, interferon-α monotherapy resulted in a small rate of SVR in patients with cirrhosis due to low virological efficacy and poor tolerance resulting in a high rate of premature interruption of treatment. Accordingly, in the studies including a subset of cirrhotic patients, SVR rates in those populations did not usually exceed 15%.

Table 3 Severe liver-related events and deaths occurring from the beginning of first treatment according to final virological response

	SVR *n* = 37	Non SVR *n* = 76	*P*
Number of patients with at least one liver-related events or death during follow-up (%)	3[1] (8.1%)	34[2] (44.7%)	0.002
Number of patients (%) with			
HCC	1 (2.7%)	24 (31.6%)	0.01
Ascites	2 (5.4%)	8 (10.5%)	0.43
Digestive haemorrhage	1 (2.7%)	4 (5.3%)	0.62
Encephalopathy	0	2 (5.4%)	0.56
Number of deaths	0	20 (26.3%)	0.002
Liver-related	0	17	
Liver failure	0	7	
GI haemorrhage	0	1	
HCC	0	9	
Non liver-related	0	3	
Suicide	0	1	
Miscellaneous	0	2	

[1]1 patient had 2 complications; [2]4 patients had 2 complications. SVR: Sustained virological response; GI: Gastrointestinal.

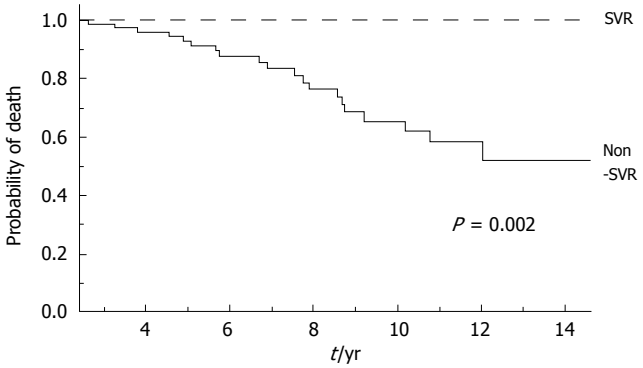

Figure 3 Incidence of death from the beginning of the first treatment according to response to antiviral treatment. SVR: Sustained virological response in dotted line; Non-SVR in full line; Log-Rank, $P = 0.002$.

Our study has several differences. Firstly, it was strictly restricted to patients with histological proven cirrhosis. This population is clearly at high risk of severe short-term complications occurrence as demonstrated by previous studies[1,26]. Secondly bitherapy using interferon-α and ribavirin was used in about 70% of cases in our study, with pegylated interferon in 38 cases. Thirdly antiviral treatment was repeated each time it was possible due to the absence of a priori contra-indication to treatment in those patients. Overall, after one (51.4%) or repeated (48.6%) antiviral treatments, 33% of our patients achieved HCV eradication, which is a rate twice higher than those obtained in previous studies.

Due to the lack of randomization, patients with SVR were obviously different from non SVR. Particularly, they were younger, more often men and significantly more frequently infected with HCV genotypes 2 or 3 than non SVR patients, as observed by Bruno *et al*[10]. Excepted male gender, these factors had been previously identified as predictive factors of SVR and it is not surprising to identify them in our study. The reduced rate of serious events in patients with SVR could result more from the presence of protective factors than from antiviral treatment. Patients with SVR were younger and this could result in a lower risk to develop complications as demonstrated for HCC occurrence. The role of genotype in spontaneous outcome of HCV-related cirrhosis although still debated does not seem to be a determinant factor in this setting and the male sex is a favouring factor of fibrosis progression and HCC occurrence. Subsequently the absence of serious event seems related to the complete response to treatment.

Several successive treatment courses have been performed in most of the patients, representing a cumulated time of treatment of 15.3 ± 9 mo. It could explain why clinical event incidence was decreased with a linear fashion from the start of treatment even if viral eradication was obtained later with further treatment. About half of SVR patients had no HCV eradication after the first course of treatment. It could seem questionable that severe events incidence have been reduced even before eradication was achieved in those patients. We postulate that it could reflect a more complete blockade of HCV replication in those patients occurring from the first course of treatment and due to higher sensitivity of virus. The fact that patients received similar duration of treatment in both groups is in favour of this hypothesis and against a non specific effect of treatment such as antifibrotic and/or antiproliferative effects of interferon.

The association between SVR and a significantly reduced number of clinical events has been previously reported[10,16,17,25]. However, this result cannot be applied to the whole population of patients with HCV-cirrhosis. In this study as in others, patients were selected and those with initial major contra-indication to treatment were excluded. These patients are probably the most severe with the higher risk of developing complications such as HCC. In addition, due to the selection of patients, the risk of death related to co morbidity was very low in our study.

Even in case of SVR, the periodic follow-up of cirrhotic patients should be recommended, particularly HCC screening as it is worth noting that the only HCC observed in SVR occurred 4.8 years after the achievement of viral clearance. In spite of a significant decrease in the incidence of HCC in case of SVR, several isolated cases of HCC were reported 4.5 to 6.6 years after the achievement of a SVR[27-30].

In conclusion, repeated antiviral therapy actually results in SVR in 33% of patients with HCV-cirrhosis. Virological cure seems to be associated with a strong decrease in the incidence of complications particularly HCC. These results are a strong argument to perform and repeat antiviral treatments in patients with compensated cirrhosis.

COMMENTS

Background

HCV cirrhosis is a life threatening disease with annual incidences of hepatocellular carcinoma (HCC), decompensation and death reaching around 3%, 4% and 3% respectively. The achievement of a sustained viral eradication (SVR) with the initially recommended 24-wk course of interferon-α monotherapy is associated with a slight preventive effect on HCC occurrence but its influence on the incidence of decompensation or death was less studied and more controversial.

Reasearch frontiers

Subsequent progresses in the field of HCV treatment (including extension of therapy to 48 wk, the combination of interferon-α with ribavirin, and the use of pegylated interferon) have created a new perspective for patients with HCV-cirrhosis because of the higher rate of SVR. Thus, the clinical long-term benefit of antiviral treatment in patients with HCV cirrhosis needed to be re-assessed.

Innovation and breakthroughs

Long-term effect of standard interferon-α plus ribavirin therapy on incidence of HCC in patients with HCV cirrhosis was recently studied in Chinese patients. Our results are the first in Western patients. In addition, death was a clinical end-point and we showed a significant reduced mortality in patients who achieved SVR.

Applications

While firm recommandations on antiviral treatment for patients with compensated HCV-cirrhosis was not made by international conferences, this is a strong argument to perform and repeat antiviral bitherapy in these patients to achieve SVR.

Terminology

Sustained virological response (SVR) is defined by the absence of detectable serum HCV RNA six months after the end of antiviral treatment and is associated with a durable viral eradication in most patients. Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and the first cause of death in patients with HCV-cirrhosis.

Peer review

The manuscript by El Barks *et al* demonstrates that antiviral treatment in patients with HCV-related cirrhosis decreases the incidence of HCC. The data provided argue for an antiviral treatment in HCV patients. The study was well performed and the data are clearly presented.

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RAPID COMMUNICATION

Pre-existing cirrhosis is associated with increased mortality of traumatic patients: Analysis of cases from a trauma center in East China

Zuo-Bing Chen, Lin-Mei Ni, Yuan Gao, Chen-Yan Ding, Yun Zhang, Xue-Hong Zhao, Yun-Qing Qiu

Zuo-Bing Chen, Lin-Mei Ni, Yuan Gao, Chen-Yan Ding, Yun Zhang, Xue-Hong Zhao, Yun-Qing Qiu, Department of Emergency Surgery, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Correspondence to: Yun-Qing Qiu, MD, Department of Emergency Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qing-chun Road, Hangzhou 310003, Zhejiang Province, China. bigzyun1@sina.com

Telephone: +86-571-87236303 Fax: +86-571-87236626
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Abstract

AIM: To determine the impact of cirrhosis on trauma patients and define the factors predicting death.

METHODS: The data on patients admitted to the trauma center from January 2000-2005 were studied retrospectively. The clinical variables were recorded and compared to identify the factors differentiating cirrhotic trauma survivors from non survivors. Child's classification criteria were derived from the reviewed charts of cirrhotic trauma patients to evaluate their predictive value in cirrhotic trauma. Trauma registry was also used to generate a trauma control group by matching for age, sex, abbreviated injury score (AIS) over the same period of time. The outcome variables compared were mortality rate, time of ICU and hospital stay. Results were expressed as mean ± SD. These data were analyzed by SPSS.11.0 statistical software. Univariate analysis was performed to identify significant medical factors for survivor and non survivors subjected to chi-square test. Fisher's exact test and Student's *t* test were performed to determine the statistical difference between cirrhotic and control groups. $P < 0.05$ was considered statistically significant.

RESULTS: Poor prognosis of traum patients was associated with one or more of the following findings: ascitcs, hyperbilirubinemia (more than 2 mg/dL), hypoalbuminemia (less than 3.5 mg/dL), and prolonged prothrombin time (more than 12.5 seconds). Although Child's classification was used to predict the outcome in cirrhotic patients undergoing portacaval shunt procedures, no significant difference was found in mortality rate as a function of Child's classification.

CONCLUSION: Cirrhosis is associated with a higher mortality, a longer time of ICU and hospital stay of trauma patients. It seems that treatment of trauma patients with pre-existing severe liver disease is a challenge to surgeons.

Key words: Pre-existing cirrhosis; Trauma; Mortality rate

Chen ZB, Ni LM, Gao Y, Ding CY, Zhang Y, Zhao XH, Qiu YQ. Pre-existing cirrhosis is associated with increased mortality of traumatic patients: Analysis of cases from a trauma center in East China. *World J Gastroenterol* 2007; 13(42): 5654-5658

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INTRODUCTION

Liver cirrhosis is the tenth leading cause of death[1]. Although cirrhosis-related deaths have decreased over the years, the impact of cirrhosis remains with approximately 30 000 deaths annually, mostly in Asian countries[2]. Cirrhotic patients often suffer from complications[3]. Cirrhosis impairs nutrition, alters response to stress, and affects the functions of other organ systems.

Trauma continues to be a major public health problem in the world. Due to the rapid economic development in China, the number of motor vehicles has increased tremendously in the past decade[4], leading to more deaths and injuries resulting from trauma. WHO predicts that trauma injuries will result in about 2.3 million deaths globally by 2020, becoming the third contributor to the global death[5]. Trauma not only causes a significant loss of life, but also results in loss of economic, medical, educational, and legal resources.

Trauma, in combination with cirrhosis in patients, brings about a unique challenge. Surgeons in trauma centers treat a variety of patients every day, but treatment of traumatized cirrhotic patients remains a challenge[6]. It has been shown that the mortality and morbidity rates increase in patients with cirrhosis undergoing elective or emergency surgery[7]. Also the degree of hepatic insufficiency is a prime factor for determining the outcome in these patients[8]. Despite the interest in the surgical outcome of cirrhotic patients, few reports are available on the outcome of cirrhotic patients after traumatic

injury. This study reviews and analyzes the data on trauma patients with pre-existing cirrhosis who were admitted to a trauma center in East China from 2000 to 2005.

MATERIALS AND METHODS

Data on more than 11 000 trauma patients (64 trauma patents with pre-existing liver cirrhosis) admitted to the Trauma Center of the First Affiliated Hospital, College of Medicine, Zhejiang University, China, from January 2000 to January 2005 were contained in their respective trauma registries. The data on patients under the ICDM-3 index for cirrhosis patients with chronic liver disease were analyzed. The trauma registry abstracts and medical records of all patients diagnosed as hepatic cirrhosis were reviewed. Diagnosis of hepatic cirrhosis was confirmed by the past medical history, clinical examination, operation findings, biopsy, and/or imaging.

The clinical variables were recorded and compared to identify the factors affecting prognosis of the patients and prolonging survival of the patients. In addition, evidence of hepatic insufficiency was evaluated by ascites, hyperbilirubinemia (more than 2 mg/dL), hypoalbuminemia (less than 3.5 mg/dL), elevated alkaline phosphatase (more than 125 st/L), serum glutamic oxaloacetic transaminase (SGOT more than 40 st/L), and/or prothrombin time (more than 12.5 s) detected at admission.

The trauma registry was also used to generate a trauma control group consisting of 86 patients by matching for age, sex, abbreviated injury score (AIS) over the same period of time. The AIS-85 scores were used because they permitted us to match patients with similar injuries. No cirrhosis or chronic liver failure was found in patients within the control group. The outcome variables compared were mortality rate, time of ICU and hospital stay.

Results were expressed as mean ± SD. The data were analyzed by SPSS.11.0 statistical software. As our sample size was too small to perform multivariate analysis, a univariate analysis was performed to identify the significant predictive factors. The data were subjected to chi-square test. To determine the statistical difference between the cirrhotic and control groups, we compared the mortality rates by Fisher's exact test. Parametric values of AIS, and time of hospital and ICU stay were compared by Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Sixty-four cirrhotic trauma patients (5.9 per 1000 trauma patients) admitted to the Trauma Center of the First Affiliated Hospital, College of Medicine, Zhejiang University were include in this study. These patients were diagnosed as cirrhosis before admission and during laparotomy for traumatic injury, respectively. The etiology of cirrhosis was related to HBV infection and alcohol in 62 and 1 patients, respectively and unidentified in 1 patient. The demographic and outcome data are listed in Table 1. Although the major causes for injury were motor vehicle accidents (MVA) (Table 2), 30.77% (4 of 13) of deaths were due to accidental fall. Other causes for injury included superficial abdominal stab wound, criminal assault. Seven patients had sustained blunt thoracic traumas including rib fracture, pulmonary contusion. Blunt abdominal trauma as evidenced by hemoperitoneum, splenic rupture, and/or liver laceration was the predominant injury in 9 patients. Pelvic fracture or limbic long-bone fracture occurred in 36 patients. Twenty-seven patients had injuries involving multiple sites. The remaining 37 patients had injuries involving a single site and two of them died (Table 3).

Table 1 Demographic and survival data on patients studied (48 males, 16 females)

(*n* = 40)	Range
Mean age = 52	31-84
Mean TS = 12	6-16
Mean ISS = 12	5-34
Surv = 87.5%	

Table 2 Mechanism of injury

Mechanism of injury	Total	Non survivors *n* (%)
Fall	13	4 (30.77)
MVA	48	3 (6.25)
Other	3	1 (33.33)

Table 3 Injury characteristics

Site	Total	Non survivors *n* (%)
Head	22	2 (9.09)
Thorax	7	2 (28.57)
Abdomen	9	3 (33.3)
Pelvis/Ext	26	1 (3.85)
Multiple	27	6 (22.22)
Single	37	2 (5.41)

The clinical or laboratory findings associated with hepatic insufficiency in this group of patients are outlined in Table 4. Ascites was confirmed during operation or radiological examination in 16 patients (6 of them died). An elevated prothrombin time of over 12.5 s was found in 15 patients (including 7 non survivors). Serum bilirubin exceeding 2 mg/dL was found in 15 patients at admission (6 of them died). The presence of any of these parameters was associated with a significant increase in mortality rate ($P < 0.05$). SGOT, alkaline phosphatase and hypoalbuminemia were elevated in many patients, but did not significantly affect their outcome.

Child's classification could predict the outcome of cirrhotic patients undergoing portacaval shunt procedures. In order to identify the predictive value of Child's classification, we retrospectively derived Child's classification criteria from the reviewed charts of cirrhotic trauma patients. The mortality of cirrhotic trauma patients according to the (retrospective) Child's classification is listed in Table 5, showing that 92.2% of our cirrhotic trauma patients corresponded to Child's class A or B. No

Table 4 Presence of HEPATIC insufficiency

Parameters of hepatic insufficiency	Total	Non survivors *n* (%)
Ascites	16	6 (37.50)[a]
SGOT > 40 μ/L	36	3 (8.33)
Alk phos. > 125 μ/L	32	3 (9.37)
Ser bili. > 2.0 md/dL	15	6 (40)[a]
Ser alb. 6 ≤ 3.0 md/dL	16	6 (37.5)[a]
PT > Control	15	7 (46.67)[a]

[a]$P < 0.05$ *vs* survivors.

Table 5 Effect of Child's classification on mortality in cirrhotic trauma patients *n* (%)

Child's classification	No. of patients (% total)	Mortality
Class A	44 (68.75)	5 (11.38)
Class B	15 (23.44)	2 (13.33)
Class C	5 (7.81)	1 (20)
Total	64 (100)	8

significant difference in mortality as a function of Child's classification was found ($P > 0.05$).

The second part of our study focused on comparing the trauma registry data between the cirrhotic trauma and control groups (Table 6). The cirrhotic trauma patients had a statistical ISS score similar to the control trauma patients. The time of ICU and hospital stay, and the mortality rate in the cirrhotic trauma group were greater than those in the control group ($P < 0.05$).

Table 6 Comparison of outcomes of trauma patients and cirrhotic trauma patients

	Cirrhosis	Control
Age (yr)	51.80 ± 13.01	48.70 ± 15.4
Percentage male	75%	76.11%
AIS	14.25 ± 8.31	13.67 ± 6.56
hospital stay	21.26 ± 5.61	8.21 ± 4.25[a]
length of ICU stay	11.24 ± 4.21	4.23 ± 1.36[a]
Mortality rate	12.53%	1.26%[a]

[a]$P < 0.05$ *vs* cirrhotic patients.

DISCUSSION

Hepatitis B is one of the most common infectious diseases in Asian countries[9], and about 10% of Chinese people have been infected with hepatitis B virus (HBV)[10]. It is estimated that about 350 million people worldwide are chronically infected with HBV, approximately 15%-40% of them are expected to develop cirrhosis and end-stage liver disease[11], which is frequently followed by hepatocellular necrosis. Cirrhosis, regardless of its etiology, inhibits the liver's response to injury. The predominant histological features are wide-spread fibrosis and nodule formation with loss of normal hepatic architecture. These changes are manifested clinically as hepatic failure and portal hypertension, the magnitude of which determines the course and prognosis of individual patients[12].

Although great progress in traumatology has been made, the number of traumatic casualties still increases[13]. In China, trauma and intoxication were the 9th, 7th and 4th leading cause of deaths in 1975, 1985 and 2000, respectively. More than 100 000 people die of traumatic injury and millions of people are injured each year in China. Furthermore, experts predict that the number of traumatic casualties will double in the 22nd century[14]. All these suggest that trauma in combination with cirrhosis is a challenge to Chinese doctors.

Adequate hepatic function is necessary in physiological response to surgery or traumatic injury[15]. The liver plays a vital role in protein synthesis, detoxification, and immune responses. In a patient subjected to surgical intervention for traumatic injury, any degree of hepatic insufficiency would diminish the liver's ability to carry out these vital metabolic functions[16]. Because of impaired cirrhotic reserves, a surgical or trauma cirrhotic patient would be at a great risk of developing complications and death may occur during the recovery period[17].

The increased risk of cirrhotic patients undergoing surgery has been well documented[18]. The Child's classification system has been used to define the surgical mortality in cirrhosis patients[19] and is moderately accurate in predicting mortality and complications of portacaval shunt surgery, but less predictive when it is applied to other types of surgery[20]. The Child's classification is mainly to classify the risk of cirrhotic patients undergoing portosystemic shunt surgery[21], showing that the degree of hepatic decompensation correlates with the rate of operative mortality in these patients. Furthermore, if therapeutic measures are taken to improve the clinical status and Child's class of cirrhotic patients before operation, the outcome of portosystemic shunting can be improved.

Child's classification criteria could not be used to classify these patients because nutritional status and response to therapy are unavailable[22]. Furthermore, changes in mental status at admission cannot be solely attributed to the etiology of cirrhosis[23]. However, hepatic insufficiency, as determined by the presence of ascites and/or elevated prothrombin time, is correlated with the outcome of these patients undergoing surgical intervention. Multivariate analysis revealed that cirrhotic trauma victims presenting with ascites, hyperbilirubinemia, or elevated prothrombin time exhibit a uniformly lower rate of survival independent of injury characteristics[24].

The impact of cirrhosis on trauma patients has recently been addressed[25]. Thirty percent of trauma patients with pre-existing liver disease have an increased risk of death and an increased time of hospital stay[26]. Tinkoff *et al*[27] have also tried to define the variables predicting the outcome of survivors and non survivors. The results of these studies suggest that trauma cirrhotic patients behave as cirrhotic patients requiring emergency surgery with similar stress and compensatory responses and that the mortality is directly related to the extent of injury.

In addition, hepatic insufficiency further diminishes survival, regardless of the injury sustained[28]. In the present study, cirrhosis was associated with a higher mortality, a longer time of ICU and hospital stay of trauma patients.

Factors predicting death are APACHE Ⅱ. ISS. RTS2,

the number of packed red blood cells transfused and organs injured, which are associated with the severity of injury[29]. Our study showed that liver insufficiency was positively associated with a poorer outcome. The lower survival and increased complication rates of cirrhotic trauma patients suggest that there is no "margin for error" in managing these patients. Thus, several management suggestions can be proposed for the improvement in cirrhotic patients with abdominal trauma[30]. It is critical to promptly diagnose and treat injuries in cirrhotic trauma patients. Since bleeding complications are frequent in cirrhotic patients, early and aggressive correction of coagulation parameters and hypothermia is crucial[31]. Poor nutrition is common in these patients and low albumin is different in survivors and non survivors. Therefore, early appropriate nutritional support should be provided. Solutions rich in branched-chain amino acids and low in aromatic amino acids can reduce hepatic encephalopathy and improve the outcome[32,33].

In conclusion, cirrhotic patients constitute a small subset of trauma patients admitted to our institution. Cirrhosis has a significantly independent adverse impact on survival of these patients. Treatment of trauma patients with severe pre-existing liver diseases remains a challenge to the surgeon.

COMMENTS

Background

Liver cirrhosis is the tenth leading cause of death. Although cirrhosis-related deaths have decreased, it leads to approximately 30 000 deaths annually, mostly in Asian countries. Trauma is a major public health problem in the world. Trauma in combination with cirrhosis brings about unique challenges and problems in patients. Surgeons in a trauma center treat a variety of patients every day, but treatment of trauma cirrhotic patients remains a challenge.

Research frontiers

Many reports have shown that mortality and morbidity rates are increased in patients with cirrhosis undergoing elective or emergency surgery. Also the degree of hepatic insufficiency is a prime factor for determining the outcome of these patients. Despite the interest in the outcome of surgical cirrhotic patients, few reports are available on their outcome.

Innovations and breakthroughs

The authors reviewed and analyzed the data on trauma patients with pre-existing cirrhosis admitted to a trauma center in East China from 2000 to 2005. This study showed that liver insufficiency was positively associated with a poorer outcome, suggesting that cirrhosis has a significantly independent adverse impact on survival of these patients.

Applications

Several management suggestions are proposed for the improvement in cirrhotic patients with abdominal trauma. It is critical to promptly diagnose and treat injuries for cirrhotic trauma patient. Since bleeding complications are frequent in cirrhotic patients, early and aggressive correction of coagulation parameters and hypothermia is crucial. Early appropriate nutritional support should be provided.

Terminology

Hepatic cirrhosis: a kind of pathological changes in liver. Hepatic cirrhosis is frequently followed by hepatocellular necrosis. Cirrhosis, regardless of its etiology, inhibits the liver's response to injury. The predominant histological features are wide-spread fibrosis and nodule formation with loss of normal hepatic architecture. These changes are manifested clinically as hepatic failure and portal hypertension, the magnitude of which determines the course and prognosis of individual patients.

Peer review

This is an interesting manuscript, showing that cirrhosis is associated with a higher mortality, a longer time of ICU and hospital stay in trauma patients. Treatment of trauma patients with severe pre-existing liver disease remains a challenge to the surgeon.

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CASE REPORT

Invasive amebiasis and ameboma formation presenting as a rectal mass: An uncommon case of malignant masquerade at a western medical center

Rosemarie E Hardin, George S Ferzli, Michael E Zenilman, Pratap K Gadangi, Wilbur B Bowne

Rosemarie E Hardin, Michael E Zenilman, Wilbur B Bowne, Department of Surgery, The State University of New York, Health Science Center of Brooklyn, NY 11203, United States
George S Ferzli, Pratap K Gadangi, Department of Surgery, Lutheran Medical Center, Brooklyn, NY 11203, United States
George S Ferzli, Department of Surgery, SUNY-Health Science Center of Brooklyn, United States
Pratap K Gadangi, Department of Surgery, Coney Island Hospital, Brooklyn NY 11203, United States
Wilbur B Bowne, Department of Surgery, Department of Veteran's Affairs, New York Harbor Health Care System, Brooklyn, NY 11203, United States
Correspondence to: Wilbur B Bowne, MD, SUNY Health Science Center @ Brooklyn, Department of Surgery, 450 Clarkson Avenue, Brooklyn, New York 11203, United States. wbbowne@earthlink.net
Telephone: +1-718-2701421 Fax: +1-718-6303706
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Abstract

A 54-year-old man presented with rectal pain and bleeding secondary to ulcerated, necrotic rectal and cecal masses that resembled colorectal carcinoma upon colonoscopy. These masses were later determined to be benign amebomas caused by invasive *Entamoeba histolytica*, which regressed completely with medical therapy. In Western countries, the occurrence of invasive protozoan infection with formation of amebomas is very rare and can mistakenly masquerade as a neoplasm. Not surprisingly, there have been very few cases reported of this clinical entity within the United States. Moreover, we report a patient that had an extremely rare occurrence of two synchronous lesions, one involving the rectum and the other situated in the cecum. We review the current literature on the pathogenesis of invasive *E. histolytica* infection and ameboma formation, as well as management of this rare disease entity at a western medical center.

Key words: Rectal ameboma; Invasive amebiasis; Ameboma; Amebic dysentery; *Entameoba histolytica*

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INTRODUCTION

Amebiasis is an infectious disease caused by the protozoan *Entamoeba histolytica*. Infection rarely, but potentially may evolve into invasive colitis and formation of ameboma, which can closely resemble colorectal carcinoma. In general, the clinical spectrum of colorectal amebiasis ranges from asymptomatic carrier to severe fulminant necrotizing colitis with bleeding and perforation[1]. As previously reported, focal ileocolonic intussusceptions from ameboma formation serving as the lead point, although rare, can occur[2].

The experience with invasive amebiasis is largely from endemic countries. From documentation dating back to 1933, to date 2970 cases of invasive amebiasis have been reported in the United States, mostly comprising recent immigrants from Mexico, Central and South America, plus Asians and Pacific Islanders[3]. With increasing patterns of immigration and travel in and out of the US, continued recognition of this disease is required. Therefore, the roles of the gastroenterologist and surgeon are to maintain a high index of clinical suspicion in patients at increased risk for this disease entity, and to distinguish this from other more common causes of gastrointestinal masses, as well as instituting appropriate therapy. In this report, diagnosis, pathogenesis and treatment of this rare clinical entity from a western medical center are reviewed.

CASE REPORT

A 54-year-old man of Middle Eastern decent, without significant medical history, presented to a community hospital complaining of rectal pain and bleeding for about 2 d. The patient described the pain as constant, without aggravating or alleviating factors. In addition, the patient complained of constipation that led to mild, crampy abdominal pain. He denied nausea, vomiting, diarrhea or fever. The patient also denied any history of weight loss. Pertinent to the patients' presentation was that he had no history of recent travel abroad, anoreceptive intercourse, or family history of carcinoma.

Physical examination revealed a healthy, well-nourished man who was normothermic with normal hemodynamic parameters. His abdominal examination was unremarkable: soft and non-tender, without any appreciable organomegaly.

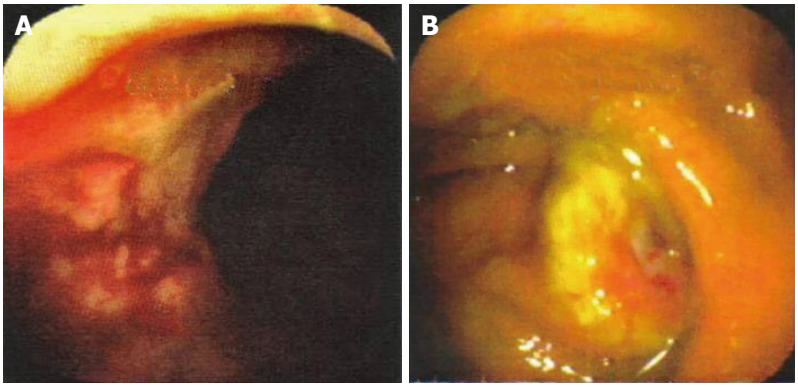

Figure 1 Endoscopy demonstrating ulcerated rectal (**A**) and cecal (**B**) lesions suggestive of carcinoma.

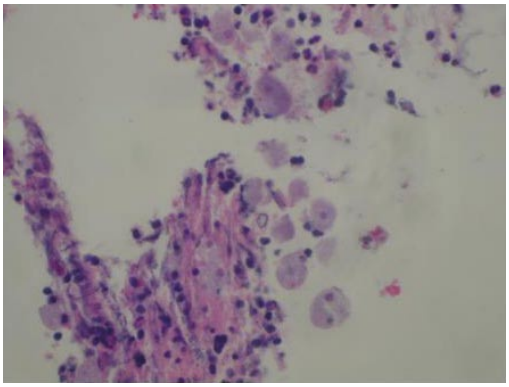

Figure 2 High-powered magnification of a hematoxylin-eosin preparation of a colonic biopsy that demonstrates trophozoites of *E. histolytica*.

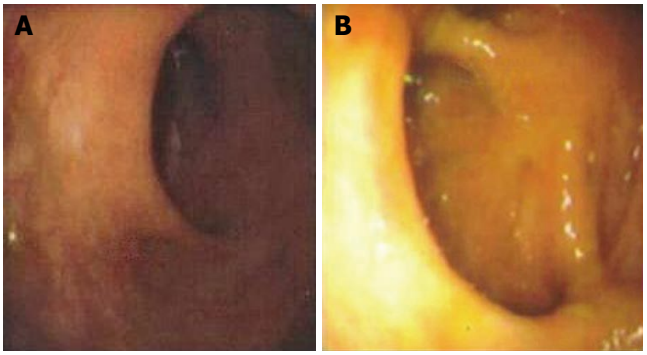

Figure 3 Follow-up colonoscopy subsequent to a completed course of antibiotic therapy, which demonstrates complete resolution of rectal and cecal lesions, with normal appearing colonic mucosa.

External anal inspection was normal. Rectal examination revealed a large, firm mass in the left lateral position, about 3 cm from the anal verge, with gross blood. Rigid proctosigmoidoscopy was performed, which revealed an ulcerated mass starting at the dentate line (Figure 1A). The surface of the lesion appeared necrotic and highly suggestive of carcinoma, which was the preliminary diagnosis. CT scan of the abdomen and pelvis revealed diffuse, non-specific thickening of the rectal wall without intra-abdominal pathology. A colonoscopy was performed that demonstrated an additional ulcerated lesion involving the cecum (Figure 1B). Both lesions were biopsied and were consistent with lymphocytic colitis. Importantly, protozoan organisms, consistent with amebiasis were identified (Figure 2). Serology for *Entamoeba histolytica* infection was also confirmed. The rectal and cecal lesions were determined to be amebomas as a result of invasive amebiasis. The patient was given a course of oral antibiotic therapy with metronidazole for 4 wk, with complete resolution of his symptoms. A follow-up surveillance colonoscopy and CT scan of the abdomen and pelvis demonstrated complete regression of both the rectal and cecal lesions (Figure 3A and B).

DISCUSSION

The intestinal protozoan parasite *E. histolytica* is the causative organism responsible for human amebiasis and amebic dysentery. Of epidemic proportions, it afflicts millions of people worldwide in developing countries, and about 40 000-100 000 people die annually from this disease[4]. Transmission is mostly by ingestion of contaminated food and water; however, venereal transmission *via* the fecal-oral route can occur[5].

Trophozoites are responsible for invasive disease and may lead to colonic mucosal ulceration. The gastrointestinal tract and liver are the two main organ systems affected by the parasite. Rarely, patients with long-standing infection develop ulcerative, exophytic, inflammatory masses that are indistinguishable from carcinomas and can become a considerable size, reportedly up to 15 cm in diameter[5]. Such lesions are referred to as amebomas. Moreover, colonic amebomas may present with synchronous amebic liver abscesses, which can also masquerade as advanced carcinoma of the gastrointestinal tract.

Amebomas result from the formation of annular colonic granulation tissue at single or multiple sites, usually within the cecum or ascending colon (Figure 4). Formation of an ameboma is an uncommon complication of invasive amebiasis. It occurs in 1.5% of all cases with invasive amebiasis[6]. Amebomas usually develop in the untreated or inadequately treated patients with amebiasis years after the attack of dysentery[5]. The current report is atypical, as our patient denied any history of antecedent symptoms suggestive of dysentery or foreign travel. Clinically, amebomas can also cause obstructive symptoms. Patients may also present with diarrhea or constipation and associated systemic symptoms, including weight loss

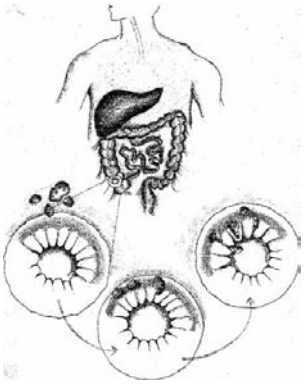

Figure 4 Pathogenesis of ameboma formation: infection is initiated by ingestion of fecally contaminated food or water containing *E. histolytica* cysts. Excystation occurs in the bowel lumen in which motile and potentially invasive trophozoites are formed. Invasion of the mucosa and submucosa may lead to colitis. Ameboma forms when severe inflammatory reaction occurs, with formation of granulation tissue that leads to a pseudotumor appearance.

and fever. In areas in which infection is prevalent, crampy abdominal pain plus a palpable mass usually suggests the diagnosis. In contrast, in western countries, a similar presentation alternatively raises suspicion of malignancy. The differential diagnosis for this clinical picture may also include inflammatory bowel disease (IBD), lymphoma, enterocolitis and intestinal tuberculosis[6]. In most cases in western countries, a diagnosis of colorectal carcinoma or IBD is considered more likely than a parasitic infection. Interestingly, there has been only a single *E. histolytica* outbreak in the United States, which occurred in Chicago in 1933 when sewage pipes contaminated tap water pipes in a hotel, which led to 800 cases of amebiasis[3].

The pathological range of amebic colitis extends from mucosal thickening with discrete ulcer formation, separated by regions of normal colonic mucosa, to diffusely inflamed and edematous mucosa. Severe forms of invasive amebiasis may progress to frank necrosis and perforation of the intestinal wall[3]. These findings are grossly similar to those found with IBD, in particular Crohn's colitis. Parasitic colitis results when the trophozoite penetrates the intestinal mucosal layer. Subsequent invasion occurs as a result of epithelial, neutrophil and lymphocyte cellular destruction by trophozoites[7].

The standard method for diagnosis of intestinal amebiasis is examination of stools by microscopy. However, the reported sensitivity of this method for identifying amebic organisms ranges from 25% to 60%[4]. False-positive results may also occur because *E. histolytica* is morphologically identical to non-pathological species, namely, *Entamoeba dispar* and *Entameba moshkovskii*[4]. Currently, more sensitive and specific methods, including antigen detection in stools and serum, and polymerase chain reaction techniques are now utilized[4].

The primary treatment for amoebic colitis is oral metronidazole therapy. Affected patients should be treated with metronidazole for 5-10 d. Longer intervals of therapy may be warranted in cases with ameboma formation, as in our patient. Treatment with metronidazole may be followed by a luminal agent such as paromomycin, iodoquinol or diloxanide furoate for 5-20 d to eradicate colonization[4]. Surgical intervention is rarely indicated except for rare instances of acute necrotizing colitis with bowel perforation, or if the patient fails to respond to anti-amebic therapy. Although amebiasis is a clinical entity rarely encountered in developed western countries, it should be included in the differential diagnosis of patients presenting with bloody diarrhea and a colon mass, with a history of dysentery along with travel to endemic areas. A high index of suspicion is required for appropriate diagnosis and treatment.

Unfortunately, there are no pathognomonic radiographic or endoscopic features suggestive of invasive ameboma formation; therefore, pathologic confirmation is crucial, as well as appropriate serology. Multiple biopsies may often be required to confirm the diagnosis. Two important caveats are essential to consider when managing patients with invasive amebomas: (1) follow up sigmoidoscopy or colonoscopy after completion of medical therapy to assure complete resolution of disease; and (2) persistent disease or incomplete regression should raise the suspicion for an underlying malignancy as the two entities may coexist[1].

In summary, the equivocal clinical symptoms (i.e., pain, bleeding and obstruction) that differentiate invasive amebiasis presenting as an ameboma from carcinoma may prove problematic. Notwithstanding, in a high-risk patient, clinical suspicion is warranted to assure appropriate diagnostic evaluation. Differentiating amebiasis from colorectal carcinoma by endoscopy with biopsy, serology, and other novel modes of antigen detection provides essential information. Therapeutic intervention with oral anti-parasitic medications rather than surgical management should then occur.

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CASE REPORT

Black esophagus with concomitant candidiasis developed after diabetic ketoacidosis

Yong Hwan Kim, Si Young Choi

Yong Hwan Kim, Si Young Choi, Department of Thoracic and Cardiovascular Surgery, Uijeongbu St. Mary's Hospital, The Catholic University of Korea, College of Medicine, Seoul 480-821, Korea
Supported by grants from the catholic university of Korea, College of Medicine
Correspondence to: Yong Hwan Kim, MD, Uijeongbu St. Mary's Hospital 65-1, Geumo-dong, Uijeongbu, Gyeonggi-do, Seoul 480-821, Korea. cmccs@naver.com
Telephone: +82-31-8203586 Fax: +82-31-8472934
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Abstract

Black esophagus is a very rare disease and its pathogenesis has been unclear. Black esophagus developed concomitantly with candidiasis after diabetic ketoacidosis has not been reported yet. We report a case who developed esophageal stricture after the treatment of black esophagus and thus balloon dilatation was performed several times but failed, hence, surgical treatment was performed.

Key words: Esophagus; Candidiasis; Diabetic ketoacidosis; Surgery

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INTRODUCTION

Black esophagus was reported in 1990 by Goldenberg *et al*[1]. Black esophagus is a rare disease and defined as a dark, pigmented esophagus at endoscopy together with histologic mucosal necrosis. The etiology remains unknown, but is most likely multifactorial, even though most reports have suggested an ischemic pathogenesis[2-5]. It caused by diabetic ketoacidosis or candidiasis, but it is very rare etiology. Here, we present a case of a patient with black esophagus developed concomitantly with candidiasis after diabetic ketoacidosis.

CASE REPORT

A 34 year old male patient with the past history of type Ⅱ diabetes transferred to the emergency room with drowsy mentality. His general condition was poor. He presented with arterial hypotension (60/30 mmHg), hyperglycemia (819 mg/dL), electrolyte imbalance (sodium 118 mEq/L and potassium 6.5 mEq/L), impaired renal function (BUN 194 mg/dL and creatinine 2.90 mg/dL), and an acid-base disorder known as metabolic acidosis (pH = 7.241 with low bicarbonate levels). Urine ketones were elevated (+++). The clinical picture suggested that patient had diabetic ketoacidosis.

Two days after admission, hematemesis was detected, Esophago-gastro-duodenoscopy (EGD) showed black and friable mucosa from 3 to 4 cm below the cricopharyngeus to the cardia (Figure 1). Biopsies of the esophagus were not obtained at this time due to concern about possible perforation.

The patient gradually recovered after the conservative treatments such as intravenous proton pump inhibitor, total parenteral nutrition, oral intake restriction and antibiotics. Six days after admission, biopsies of the esophagus were obtained with EGD and revealed submucosal necrosis and candidiasis. After conservative treatments including antifungal agents (fluconazol 200 mg for seven days), the patient was discharged on the thirtieth day of admission. Subsequently, after 2 months, esophagus stricture was developed. Balloon dilation was performed 3 times during 6 months, but it was not improved (Figure 2A), and thus subtotal esophagectomy and esophagogastrostomy were performed (Figure 2B), and on the eighteenth day of surgery, he was discharged without complication.

DISCUSSION

Black esophagus was reported in 1990 by Goldenberg *et al*[1]. Black esophagus is a rare disease, and the incidence detected by EGD has been reported to be 0.01%-0.2%[2-5]. It is developed preferentially in the male (81%), and it has been reported to occur predominantly in the elderly[4].

The pathogenesis of black esophagus remains undefined and is probably a combination of an low systemic perfusion, gastric outlet obstruction, and malnutrition[2-5].

Several etiologies have been suggested including ischemia, lye ingestion, microbial infection related to a nasogastric tube, anticardiolipin antibody syndrome, herpes simplex esophagitis, diabetic ketoacidosis, severe vomiting, acute gastric outlet obstruction with massive gastroesophageal reflux, *Lactobacillus acidophilus* infection, acute caustic injury from detergent in the endoscope, antibiotics, Stevens-Johnson syndrome, and hypothermia[2,3,5]. Among 88 cases of black esophagus reported until now,

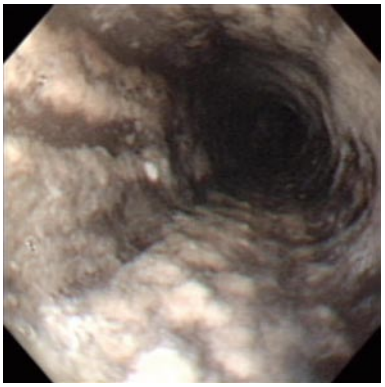

Figure 1 Endoscopy shows that esophagus is circumferentially black and friable.

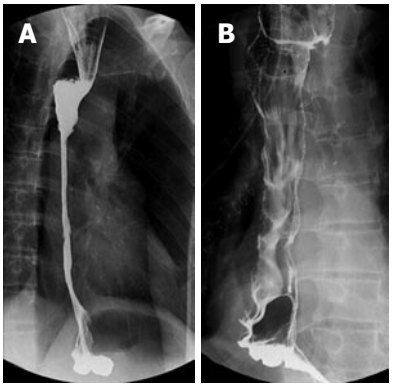

Figure 2 Barium swallow study. **A**: Stricture formation at middle esophagus; **B**: Improvement of the stricture was seen after surgical treatment.

cases whose etiology was diabetic ketoacidosis were 3 cases[4], cases whose etiology was fungal infection were 2 cases[5,6]; however, reports showing diabetic ketoacidosis and candidiasis concomitantly have not been reported.

Most patients are symptomatic, and the most common symptoms are upper gastrointestinal bleeding such as hematemesis, coffee-ground vomiting and melena[2-4].

The diagnosis is based on typical endoscopic appearance and on histopathological findings after the exclusion of true necrosis and ulceration. It shows the finding of the diffusely black esophageal mucosa, and in most cases it disappears suddenly in the Z-line. Histological findings of esophagus biopsy show necrotic debris, and it may show the finding of the necrotic mucosa without the viable epithelium, and occasionally, the submucosa and up to the muscularis propria, although rare, may be involved[2,4]. This case showed that submucosal involvement.

The prognosis of black esophagus is very poor, and its mortality reaches up to 31.8%-50%, although most of the deaths were caused by underlying illnesses[2-4]. Death secondary to esophageal necrosis occurs in less than 6% of cases[4]. Therapeutic modalities are not standardized, but most authors support a conservative approach by correcting the underlying disease, intravenous proton pump inhibitor, total parenteral nutrition, and oral intake restriction[2-5].

The major complications of black esophagus are esophageal stenosis and stricture formation, and they occur in 10.2%-15% of patients. Repeated dilation is required in most patients[2,4,5]. In our case, repeated dilation was not effective, and thus surgical treatment was performed. Other complications are esophageal perforation, mediastinitis and abscess formation, which occurs in approximately 6% patients[4].

Surgical treatments for black esophagus have been reported in 5 cases[4], nevertheless, all 5 cases were performed surgery for early complications such as esophageal perforation and our case is the first case of black esophagus performed surgical treatment for the late complication esophageal stricture.

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CASE REPORT

Giant submucosal lipoma located in the descending colon: A case report and review of the literature

Li Jiang, Li-Sheng Jiang, Fu-Yu Li, Hui Ye, Ning Li, Nan-Sheng Cheng, Yong Zhou

Li Jiang, Li-Sheng Jiang, Fu-Yu Li, Hui Ye, Ning Li, Nan-Sheng Cheng, Yong Zhou, The First Department of General Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China
Correspondence to: Professor Li-Sheng Jiang, The First Department of General Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China. jls339@126.com
Telephone: +86-13880900287
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Abstract

Colonic lipoma is an uncommon tumor of the gastrointestinal tract. Most cases are asymptomatic, with a small tumor size, and do not need any special treatment. However, we encountered one patient with a giant submucosal lipoma, with a maximum diameter of 8.5 cm, which exhibited symptoms such as intermittent lower abdominal pain, changes in bowel habits with passage of fresh blood and mucus per rectum, abdominal distension, anorexia and weight loss. Unfortunately, the possibility of colonic malignancy could not be precluded and left hemicolectomy was planned. The exact diagnosis of this special case was accomplished by intraoperative pathology. In the end, local resection was performed instead of left hemicolectomy. To the best of our knowledge, colonic lipoma exceeding 8 cm in diameter has not been previously reported. We, therefore, present this case and discuss age and sex factors, clinical and histopathological findings, diagnostic methods and treatment by reviewing the available literature, to serve as a reminder that colonic lipoma can also exist in patients with significant symptoms. In addition, intraoperative pathology should be investigated in those doubtful cases, so as to guide the exact diagnosis and treatment plan.

Key words: Colon; Lipoma; Age; Diagnosis; Therapy

Jiang L, Jiang LS, Li FY, Ye H, Li N, Cheng NS, Zhou Y. Giant submucosal lipoma located in the descending colon: A case report and review of the literature. *World J Gastroenterol* 2007; 13(42): 5664-5667

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INTRODUCTION

Lipoma of the colon is a rare condition which may be detected incidentally at colonoscopy, surgery or autopsy[1-3]. Most colonic lipomas are asymptomatic and need no treatment, whereas lesions exceeding 2 cm in diameter do produce symptoms[1,3-5]. Large colonic lipomas are usually mistaken for more serious pathology as a result of their rarity and variable presentation. Although the nosopoietic location of colonic lipoma usually varies with different cases, the commonest site is the ascending colon[2,4]. To the best of our knowledge, colonic lipoma exceeding 8 cm in diameter has not been reported previously. We report here, a patient with a giant lipoma, whose maximum diameter reached 8.5 cm, located in the submucosa of the descending colon, and review the literature regarding colonic lipoma.

CASE REPORT

A 42-year-old man presented to us in February 2007 with a 3-wk history of intermittent lower abdominal pain. One month previously, he experienced changes in bowel habits (7-10 times daily), with passing fresh blood and mucus per rectum, abdominal distension, anorexia and weight loss. A routine colonoscopy had been performed in another hospital 15 d previously, which revealed a yellowish, 7.5-cm diameter, spherical polypoid lesion arising from the lateral wall of the descending colon (Figure 1). The related biopsy revealed a lot of inflammatory necrotic tissue and a glandular cell with mildly atypical hyperplasia. Physical examination revealed mild tenderness in the left lower quadrant. Routine blood tests were normal except for mild anemia. Abdominal ultrasonography indicated a space-occupying mass of the lower abdomen. Considering the uncertain diagnosis, we suggested that colonoscopy or computed tomography (CT) should be performed again in our hospital, but this was refused by the patient. In view of his age, symptoms and related examinations, the possibility of colonic malignancy could not be precluded, and left hemicolectomy was subsequently planned.

At laparotomy, an 8.5 cm × 7 cm × 6.5 cm yellowish polypoid lesion, associated with numerous areas of ulceration on its surface, was seen arising from the descending colon. The lesion almost obstructed the whole lumen, and resulted in dilatation of the proximal colon. However, to our surprise, such a large lesion was only located on the submucosa, without invasion of the serosa. Subsequently, microscopical examination confirmed that this lesion was

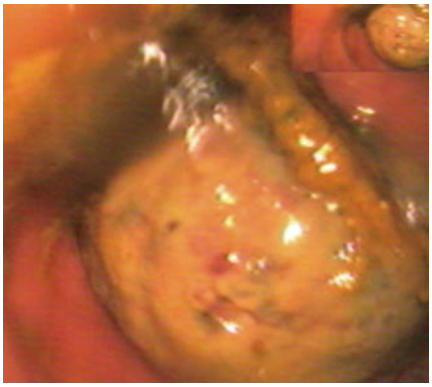

Figure 1 Colonoscopy showed a yellowish, spherical, polypoid lesion, with a lot of inflammatory necrotic tissue and numerous areas of ulceration on its surface, which arose from the lateral wall of the colon. The lesion almost obstructed the whole lumen.

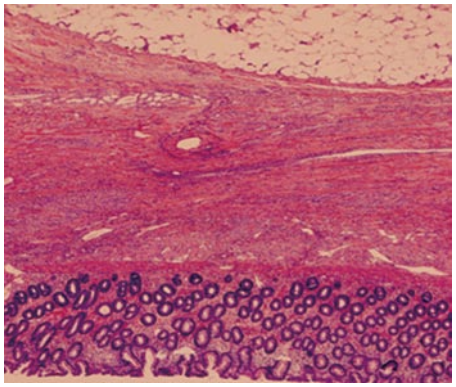

Figure 2 Histopathology showed that the lesion was located in the submucosa, of adipose origin, and was complicated with necrotic tissue and granulation on its surface (HE, × 100).

Table 1 Summary of the clinicopathologic features about treated lipomas in reviewed literature

	No	Sex		Mean age (yr)	Signs and symptoms				Pathology	Location				MMD (cm)
		M	F		AP	BPR	AIBH	anemia		AC	TC	DC	SC	
Ref.1	4	1	3	52	2	2	2	0	SL	1	0	1	2	2.5
Ref.2	3	1	2	51	3	0	0	0	SL	2	1	0	0	6.3
Ref.3	6	2	4	72.5	1	3	1	2	SL	3	0	0	3	2.9
Ref.4	8	4	4	53.3	3	3	1	1	SL (7) MSL (1)	6	1	0	1	3.7
Ref.6	5	1	4	62	3	5	1	0	SL	1	1	2	1	5
Ref.7	1	0	1	71	1	0	1	0	SL	1	0	0	0	5
Ref.8	3	0	3	71	0	3	0	0	SL (2) MSL (1)	1	0	0	2	2.2
Ref.9	2	1	1	56.5	0	1	1	0	SL	0	0	1	1	4
Present series	1	1	0	42	1	1	1	1	SL	0	0	1	0	8.5
Combined data	33	11	22	59.7	14	18	8	4	SL (31) MSL (2)	15	3	5	10	3.8
Percentage (%)		33.3	66.7		42.4	54.5	24.2	12.1	SL (93.9) MSL (6.1)	45.5	9.1	15.2	30.3	11.5

M: Male; F: Female; AP: Abdominal pain; BPR: Bleeding per rectum; AIBH: Alteration in bowel habits; AC: Ascending colon; TC: Transverse colon; DC: Descending colon; SC: Sigmoid colon; MMD: Mean maximum diameter; SSL: Simple submucosal lipoma; MSL: Multiple submucosal lipoma.

only located in the submucosa, and originated from adipose tissue, but it had necrotic tissue and ulcerations on its surface (Figure 2). Finally, the diagnosis was confirmed to be one of submucosal lipoma of the descending colon, and local resection was performed. The patient recovered and was discharged 12 d postoperatively.

DISCUSSION

Lipoma of the colon is an uncommon tumor of the gastrointestinal tract, and belongs to the group of benign non-epithelial tumors. As reported at autopsy, the incidence of colonic lipoma ranges from 0.035 to 4.4%[4]. In general, colonic lipomas do not cause symptoms and, therefore, are usually detected incidentally during colonoscopy, surgery and autopsy. However, a minority of lipomas can cause symptoms when the lesion is large, especially for those with a diameter > 2 cm[1,3-5]. To the best of our knowledge, colonic lipoma with a maximum diameter of 8.5 cm, associated with significant symptoms, has not been previously reported.

The clinicopathologic features of symptomatic lipomas are reviewed in the literature[1-4,6-9] and are summarized in Table 1. Thus, we can conclude that the most common signs and symptoms include abdominal pain (42.4%), bleeding per rectum (54.5%) and alteration in bowel habits (24.2%). With respect to sex distribution, there is a female predominance (66.7%), while other authors have found a nearly equal sex distribution[10]. The most common age is the fifth or sixth decades of life. As for its location, the most typical site for solitary colonic lipoma is the ascending colon (45.5%), whereas the lesion in our report was located in the descending colon. From Table 1, we can see that a solitary lesion is usually found in most cases; by contrast, multiple lesions occur in 6.1% of cases.

Microscopically, colonic lipomas are usually located in the submucosa, and numerous fibra intervals (Figure 3) can be observed in adipose tissue, resulting in the lobulated appearance of lipoma. Furthermore, varying degrees of fat necrosis, granulation and ulceration may be found on the surface of relatively large lipomas.

With the widespread application of colonoscopy, small lesions are found incidentally, and their diagnosis and treatment are mainly dependent on endoscopy[1-3]. However, large colonic lipomas are often mistaken for more serious pathology, as a result of their rarity and variable presentation. Therefore, more attention should be paid to how to increase the rate of preoperative diagnosis. Clinical fea-

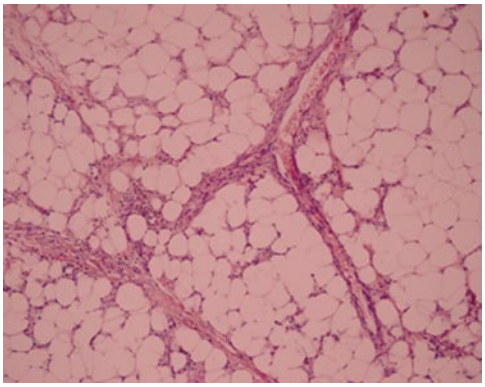

Figure 3 Histopathology showed the numerous fibra intervals in the adipose tissue (HE, × 200).

tures are still important, especially for those large lesions. Our patient with an 8.5 cm × 7 cm × 6.5 cm lesion should have presented with the appearance of complete intestinal obstruction. However, to our surprise, he did not present as an emergency with significant symptoms. Several factors may have contributed to this phenomenon. One potential explanation is the slow growth rate of colonic lipoma. Another, and perhaps more likely explanation is the long-standing obstruction caused by the lesion, which results in proximal colonic dilatation.

Although imaging findings may be less specific, they have still contributed to the preoperative diagnosis. Barium enema may demonstrate a filling defect, and the lesion may exhibit a lobulated appearance[4,6], but this phenomenon is non-specific and the lesion can be mistaken for another type of neoplasm, although water enemas are thought to improve the contrast with radiolucent fat[11]. For large colonic lipomas and acutely ill patients, CT and magnetic resonance imaging are the preferred methods because their imaging characteristics are relatively typical for adipose tissue, and they provide a rapid diagnosis[2,6].

Since most lipomas are submucosal, colonoscopy can provide direct visualization and pathologic examination via biopsy forceps. Thus, preoperative diagnosis mainly depends on colonoscopy. Typical lipomas appear as smooth, spheroidal, slightly yellowish polyps of variable size, with or without a pedicle[3]. In addition, three signs may contribute to the diagnosis, including "cushion sign"[12] (probing the polyp with a closed biopsy forceps will often yield a pillow-like indentation), "tenting effect"[12] (grasping the overlying mucosa with the biopsy forceps presents a tent-like appearance), and the "naked fat sign"[13] (biopsies may result in an extrusion of yellowish fat). Although colonoscopy is reliable for the diagnosis of the usual type of lipoma, it is more difficult for diagnosis of those lesions with an atypical, callous or ulcerated shape. In our case, the necrotic mucosa and numerous areas of ulceration that existed on the tumor surface, together with a relatively hard texture, may have confused the above-mentioned signs, therefore leading to the subsequent misdiagnosis. In addition, biopsy by colonoscopy may provide limited help for the diagnosis of some large lesions, because it cannot obtain adequate tissue. If the adipose tissue lies beneath the normal or ulcerated mucosa, it is not likely to be diagnostic; furthermore, if biopsy yields benign tissue, it is not possible to completely exclude the possibility of malignancy. The exact diagnosis still mainly relies on an intra- or postoperative pathology examination.

Many therapeutic interventions have been tried for the treatment of colonic lipoma, which have varied from hemicolectomy to segmental resection and local excision, according to the correct preoperative diagnosis and intraoperative findings. With the advancement of colonoscopy, endoscopic cautery snare resection of colonic lipomas has become popular and has been proven to be a safe therapeutic method, especially for small lesions[1,3,4,6,7,14]. However, various views with regard to endoscopic removal of large lipomas have been reported. Some studies have demonstrated that removal of lipomas $\geqslant$ 2 cm in diameter is associated with a greater risk of perforation[3,15,16]. On the contrary, some authors have reported that large pedunculated and large sessile lesions can be removed without perforation[6,7]. Kim *et al*[1] have performed endoscopic removal of lipoma with a maximum diameter of 3.8 cm, assisted by injection of saline solution with or without epinephrine into the submucosa beneath the lesion, with no complications. Bar-Meir *et al*[7] have described the safe endoscopic removal of a very large 5-cm lipoma. In addition, the feasibility of slow mechanical transection of a large colonic lipoma (4 cm) with an endoloop ligation technique has been demonstrated by Raju *et al*[15], whereas this novel technique may require application of additional loops several weeks later. The removal of colonic lipoma with the assistance of laparoscopy has also been reported[17].

However, on the basis of our case and the published literature, we think that surgical removal should be the preferred choice for the following indications: (1) lipoma with a diameter of > 4 cm, with a sessile appearance or limited pedicle; (2) unclear preoperative diagnosis; (3) lesions with significant symptoms, especially the appearance of intussusception; (4) involvement of the muscular layer or serosa; and (5) lesion cannot be resected radically under colonoscopy. Although colonic lipoma is a benign tumor, intraoperative frozen sections are required to ensure negative surgical margins, which can guide the choice of surgical approach. As far as our patient was concerned, left hemicolectomy would have been performed instead of local resection, if we had not taken a frozen section intraoperatively. Overall, intraoperative pathology is the most important examination for doubtful cases of colonic lipoma, which can also assist in guiding the exact diagnosis and treatment planning.

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Regensburg University Medical Center, Internal Medicine I, Franz Josef Strauss Allee 11, 93042 Regensburg, Germany

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Paolo Del Poggio, Dr
Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

Wendy Michelle Mars, PhD
Department of Pathology, University of Pittsburgh, S-411B South Biomedical Science Tower Pittsburgh, PA 15261, United States

Gianluigi Giannelli, MD
Dipartimento di Clinica Medica, Immunologia e Malattie Infettive, Sezione di Medicina Interna, Policlinico, Piazza G. Cesare 11, 70124 Bari, Italy

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211 Woodland Road, Kentfield, California 94904, United States

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Department of Internal Medicine and University Service of Gastrohepatology, University Hospital Center " Mother Theresa", Medical Faculty of Tirana, Tirana, Albania

Luigi Bonavina, Professor
Department of Surgery, Policlinico San Donato, University of Milano, via Morandi 30, Milano 20097, Italy

Shinji Tanaka, Director
Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

Richard A Kozarek, MD
Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

Jia-Yu Xu, Professor
Shanghai Second Medical University, Rui Jin Hospital, 197 Rui Jin Er Road, Shanghai 200025, China

Seyed-Moayed Alavian, Professor
P.O.Box: 14155-3651, Tehran, Iran

Francesco Negro, MD
Divisions of Gastroenterology and Hepatology and of Clinical Pathology, Hôpital Cantonal Universitaire, 24 rue Micheli-du-Crest, CH-1211 Genève 14, Switzerland

Florian Graepler, Dr
Department of Gastroenterology, Hepatology and Infectious Diseases, University Hospital Tuebingen, Otfried-Mueller-Str. 10, D-72076 Tuebingen, Germany

Murray B Resnick, MD, PhD
Department of Pathology, Rhode Island Hospital, 593 Eddy street, Providence RI 02903, United States

Pietro Invernizzi, Dr
Division of Internal Medicine, Department of Medicine, Surgery, Dentistry, San Paolo School of Medicine, University of Milan, Via Di Rudinfi 8, 20142 Milan, Italy

Özlem Yilmaz, PhD, Associate Professor of Microbiology
Dokuz Eylul University, School of Medicine, Department of Microbiology and Clinical Microbiology, Inciralti 35340, Izmir, Turkey

Nikolaus Gassler, Professor
Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Chung-Mau Lo, Professor
Department of Surgery, The University of Hong Kong, Queen Mary Hospital, No.102 Pokfulam Road, Hong Kong, China

Joseph Daoud Boujaoude, Assistant Professor
Department of Gastroenterology, Hotel-Dieu de FranceHospital, aint-Joseph University, Beirut 961, Lebanon

Gerardo Nardone, MD, Associate Professor
Department of Clinical and Experimental Medicine, University of Naples Federico II, Via Pansini 5, Napoli 80131, Italy

Richard A Rippe, Dr
Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Einar Stefan Björnsson, Professor
Department of Internal Medicine, Section of Gastroenterology and Hepatology, Sahlgrenska University hospital, Med pol II, SE-413 45 Gothenburg, Sweden

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Department for Mucosal Immunity, Helmholtz centre for Infection Research, Inhoffenstrasse 7, Braunschweig 38124, Germany

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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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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Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com

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EDITORIAL

Specifically targeted antiviral therapy for hepatitis C virus

Anna Parfieniuk, Jerzy Jaroszewicz, Robert Flisiak

Anna Parfieniuk, Jerzy Jaroszewicz, Robert Flisiak, Department of Infectious Diseases, Medical University of Bialystok, Zurawia St 14, Bialystok 15540, Poland
Correspondence to: Robert Flisiak, Department of Infectious Diseases, Medical University of Bialystok, Zurawia St 14, Bialystok 15540, Poland. flisiakr@priv.onet.pl
Telephone: +48-85-7416921 Fax: +48-85-7416921
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Abstract

Hepatitis C virus (HCV) infection affects 180 million people worldwide with the predominant prevalence being infection with genotype 1, followed by genotypes 2 and 3. Standard anti-HCV therapy currently aims to enhance natural immune responses to the virus, whereas new therapeutic concepts directly target HCV RNA and viral enzymes or influence host-virus interactions. Novel treatment options now in development are focused on inhibitors of HCV-specific enzymes, NS3 protease and NS5B polymerase. These agents acting in concert represent the concept of specifically targeted antiviral therapy for HCV (STAT-C). STAT-C is an attractive strategy in which the main goal is to increase the effectiveness of antiviral responses across all genotypes, with shorter treatment duration and better tolerability. However, the emergence of resistant mutations that limit the use of these compounds in monotherapy complicates the regimens. Thus, a predictable scenario for HCV treatment in the future will be combinations of drugs with distinct mechanisms of action. For now, it seems that interferon will remain a fundamental component of any new anti-HCV therapeutic regimens in the near future; therefore, there is pressure to develop forms of interferon that are more effective, less toxic, and more convenient than pegylated interferon.

Key words: Hepatitis C virus; Chronic hepatitis C; Polymerase inhibitors; Protease inhibitors; Cyclophilin B inhibitors

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INTRODUCTION

Hepatitis C virus (HCV) infection affects 180 million people worldwide, about 3% of the world's population. Globally, 3-4 million persons are newly infected each year, with the predominant prevalence being infection with genotype 1, followed by genotypes 2 and 3. The other genotypes, 4, 5, and 6, have specific geographical distributions. The acute phase of infection is asymptomatic in the majority of cases, but leads to chronic infection in about 70%-80% of affected individuals[1,2]. This results in progressive liver disease and eventually liver cirrhosis, with increased risk of hepatocellular carcinoma[3].

HCV is an enveloped virus belonging to the Flaviviridae family. The virion consists of an outer envelope composed of E1 and E2 proteins covering the nucleocapsid, with a single-stranded RNA genome (Figure 1). The HCV genome encodes structural proteins that form the capsid and envelope, as well as non-structural proteins required for virus replication. The latter are potential targets for new drugs aimed at directly affecting HCV. The non-structural components include proteases and helicase (NS2, NS3 and NS4A), a protein responsible for anchoring the replication complex to intracytoplasmic membranes (NS4B), the RNA-binding protein (NS5A), and RNA-dependent RNA polymerase (NS5B). After entering the cell, HCV RNA is released into the cytoplasm. The genome is directly translated by host enzymes. The HCV polyprotein is subsequently processed by the host and viral proteases into structural and non-structural proteins, including NS5B, which is crucial for HCV replication. There are two viral proteases, NS2-3 and NS3. NS2-3 is responsible for cleavage at the NS2/3 site. NS3 catalyses cleavage at four sites: NS3/4A, NS4A/B, NS4B/5A and NS5A/B. The proteolytic activity of NS3 protease is significantly enhanced by heterodimerization with its cofactor NS4B[4].

CURRENT TREATMENT OPTIONS

The therapy currently regarded as the standard consists of pegylated interferon injections administered once weekly, along with daily oral ribavirin. This combination exerts synergistic antiviral effects, although it is efficient in only about 50% of treated individuals. The most important predicting factor appears to be the HCV genotype. Sustained viral response (SVR) expressed as an absence of detectable serum HCV RNA 24 wk after completion of therapy is achieved in only 33%-42% of patients with genotype 1, but in about 90% of those with genotypes 2 and 3[5]. There are patients who do not achieve an SVR due to unresponsiveness or relapse after treatment, as well as those who lack tolerance to adverse events that occur during treatment. Therefore, there is a need for new therapeutic strategies with higher efficacy, shorter

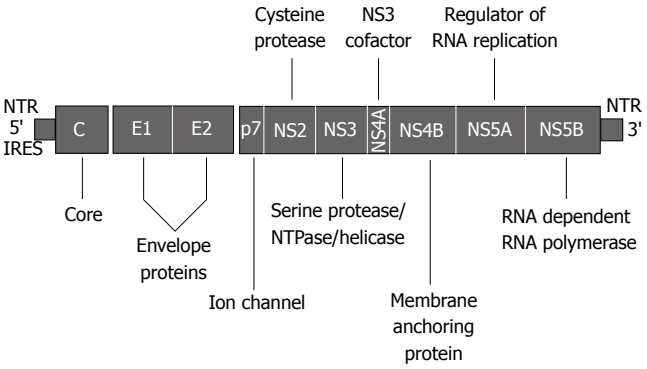

Figure 1 Organization of the HCV genome (adapted from Appel *et al*[4]).

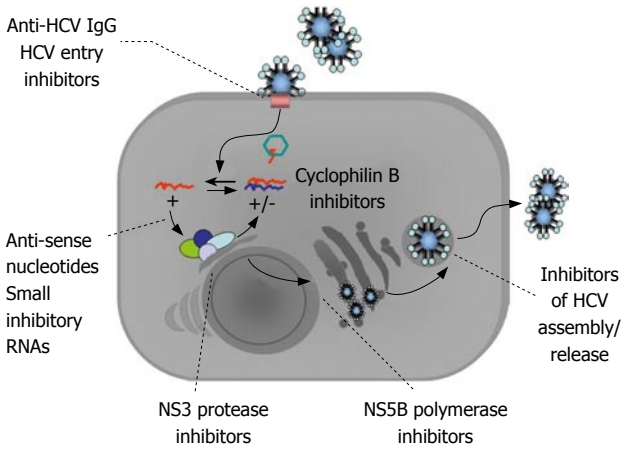

Figure 2 Potential targets for new anti-HCV agents.

treatment duration, convenient routes of administration and favorable side-effect profiles.

The standard anti-HCV therapy currently aims to enhance natural immune responses to the virus; whereas, new therapeutic concepts directly target HCV RNA (anti-sense nucleotides, small inhibitory RNA) and viral enzymes (protease and polymerase inhibitors), or influence host-virus interactions (cyclophilin inhibitors, bavituximab, inhibitors of viral entry) (Figure 2)[6-9]. These findings essentially came from attempts to create an experimental model for studies of the molecular details of the virus and HCV infection of cells. The development of the replicon system and pseudoviruses has greatly contributed to the current knowledge of HCV replication. However, a highlight in recent studies on HCV has been the establishment of a complete cell culture replication system that enables further research into the HCV lifecycle and novel antivirals.

NOVEL ANTIVIRAL AGENTS

Novel treatment options now under intensive development are focused on the inhibitors of HCV-specific enzymes, NS3 protease and NS5B polymerase. The actual protease inhibitors under clinical development are telaprevir and SCH 503034. Among the polymerase inhibitors, several drugs have reached the clinical stage. These include valopicitabine, R1479, HCV-796 and BILB1941. Such agents acting in concert represent the concept of specifically targeted antiviral therapy for HCV (STAT-C). The principle of this new therapeutic strategy is the achievement of higher rates of efficacy, shortened treatment duration, improved tolerability and adherence, oral administration and the possibility for use in special populations (e.g., those with contraindications to interferon). However, the emergence of resistance mutations that limit the use of these compounds in monotherapy complicates the regimens. Thus, a predictable scenario for treatment of HCV in the future will be combinations of drugs with distinct mechanisms of action. Interferon, however, appears pivotal for any new therapeutic regimens.

INTERFERONS IN DEVELOPMENT

Pegylated interferon underpins the current standard of care therapy for HCV. Its immunomodulatory activities with positive pharmacokinetics significantly improve the efficacy of anti-HCV regiments, although the effectiveness of antiviral treatment remains unsatisfactory, especially as regards genotype-1 infections. Another limitation is the frequent occurrence of adverse events, which greatly influence adherence to the treatment regimen. Therefore, there is pressure to develop forms of interferon that are more effective, less toxic, and more convenient than pegylated interferon. Phase 1 clinical trials are in progress with oral forms of interferon (Belerofon; oral interferon alpha). Another goal in the field of interferon development is to obtain forms with a longer half life, which will allow a reduction in the number of injections required and lengthen the period between injections. These agents [IL-29 (PEG-interferon lambda), Belerofon Interferon (long-acting), BLX-883 (Locteron), and Medusa Interferon (Multiferon)] are mostly in the first or second phase of their clinical development. Omega interferon, which is in phase Ⅱ development, is used in an implantable infusion pump that releases a steady amount of interferon for about 1 mo[10]. Albinterferon (IFN-α2b genetically fused to human albumin) and interferon β-1a (REBIF) have reached phase Ⅲ development. Albinterferon is a protein that combines the antiviral properties of IFN-α2b with the prolonged half-life of human serum albumin. Clinical trials have shown that albinterferon administered every 2 wk exerts an antiviral efficacy comparable to that of Peg-IFN-α2a, with a similar frequency of occurrence of adverse events and laboratory abnormalities[11]. Consensus interferon (Infergen) administered daily is currently in phase Ⅳ clinical trials, and research evaluating its usefulness in treatment of prior non-responders is ongoing, with promising interim results[12,13].

PROTEASE INHIBITORS

Telaprevir (VX-950), an investigational oral selective inhibitor of HCV NS3/4A protease[14], appears to be the one of most advanced therapeutic agents that specifically target the HCV lifecycle. Clinical phase Ⅱb studies utilizing VX-950 are ongoing. These include the PROVE

1 and PROVE 2 trials that have enrolled 260 and 320 treatment-naïve HCV genotype 1 patients, respectively, and the PROVE 3 study in patients with HCV genotype 1, previously treated by standard of care therapy (at least one prior course of peginterferon with ribavirin).

The phase Ⅰb studies showed that VX-950 had potent anti-HCV activity in monotherapy, which led to a rapid decline in HCV RNA of about 3 log within 3 d in all dosage groups (450 mg q8h, 750 mg q8h and 1250 mg q12h), both in genotype 1 treatment-naïve patients and non-responders to prior treatment. In the optimal dose group, 750mg q8h, the highest plasma concentration was achieved with a 4.4 log reduction in HCV RNA load at d 14. When sub-optimal doses were applied, maximal HCV RNA reduction was seen after 3-7 d treatment, with any following increase in viral load being related to the selection of HCV variants less sensitive to VX-950[15,16]. Moreover, other studies have reported that the abovementioned HCV RNA fluctuations, as well as alanine aminotransferase (ALT) levels during treatment correlated with the concentration of neopterin - a monocyte/macrophage-derived factor that can be considered an indicator of inflammatory activity during the course of chronic C hepatitis[17,18]. Furthermore, the ultrasound evaluation of perihepatic lymph nodes (PLNs) in HCV-infected individuals undergoing treatment with telaprevir alone showed a significant reduction in PLN volume after 14 d therapy. This suggests that VX-950 may have the ability to reduce inflammatory activity in liver tissue; however, this research is limited by a lack of objective parameters for inflammatory activity, such as histopathological evaluation[19].

The HCV NS3 protease diversity determined before administration of VX-950 appears to have no predictive impact for the effectiveness of antiviral responses[20]. Whereas, identification of HCV NS3 protease variants after 14 d treatment demonstrates the emergence of mutations that implicate varying decreases in sensitivity to VX-950, low-level resistance characterized by V36A/M, T54A, R155K/T, and A156S mutations, and high-level resistance determined by the emergence of A156V/T, V36A/M-R155K/T, and V36A/M-A156V/T variants. Each dosage group has a distinct mutation pattern that results from the different levels of drug pressure on the variants selected. It has been suggested that these drug-resistant variants pre-exist at a minimal frequency before the introduction of treatment, because of the low fidelity of HCV polymerase and the high rate of HCV replication. Resistant variants appear to be less fit and have lower replication rates compared to wild type; therefore, they may not be detected prior to treatment[21,22].

Further studies of VX-950 administered with peginterferon, with or without ribavirin, have confirmed the advantageous effect of combination therapy on virus selection. Data presented at the American Association for the Study of Liver Diseases (AASLD) Meeting in 2006 demonstrated a 5.5 log HCV RNA drop when VX-950 was combined with peginterferon α2a *vs* a 1 log drop in those receiving peginterferon alone, and a 4 log decrease in monotherapy with VX-950 at d 14 of the study[23]. With regard to selection of the resistant variant, this study provides evidence of the suppressing effect of peginterferon when it is included in a combination therapy regimen, or is applied as a follow-on after discontinuation of VX-950, thus indicating that VX-950-resistant variants remain sensitive to the standard care therapy. This observation is consistent with *in vitro* research confirming the decreased replication capacity of resistant variants while the sensitivity to interferon is fully retained[22]. Interestingly, in a few patients receiving VX-950 alone, the higher level resistant variant A156V/T emerged, but was subsequently suppressed by therapy with VX-950 followed by peginterferon and ribavirin. In this study, all patients receiving peginterferon and ribavirin subsequent to 14 d treatment with VX-950 had undetectable HCV RNA at the end of wk 24. However, the discontinuation of therapy at that point in individuals with undetectable HCV RNA at wk 12, resulted in relapses in two of the four patients from the VX-950 monotherapy group, and one of six from the VX-950 with peginterferon group, which showed the advantages of combination therapy over monotherapy[23,24]. The interim results after 12 wk of the PROVE 1 study, the first major phase Ⅱ clinical trial to evaluate VX-950, are now available. Analysis shows a definitely higher incidence of undetectable HCV RNA [limit of detection (LOD) 10 IU/mL] at wk 12 in patients receiving VX-950 in combination with peginterferon and ribavirin, in contrast to those receiving standard therapy (88% *vs* 52%). The frequency of adverse events was comparable in the telaprevir-treated and control groups. However, discontinuation due to adverse events was higher in the telaprevir than in the control groups, 9% *vs* 3%. The adverse events most frequently reported in the telaprevir group included rashes (3%), gastrointestinal disorders and anemia. The incidence of serious adverse events in the telaprevir groups was about 3% compared to 1% in the control[25]. Further research on telaprevir that aimed to assess its activity against the NS3/4A proteases of HCV genotypes 2, 3 and 4 was presented at the European Association for the Study of the Liver (EASL) Meeting in 2007. *In vitro* studies have demonstrated that the VX-950 activity against genotypes 2a, 2b, 3a and 4a is similar to that for genotypes 1a or 1b. Moreover, NS3/4A protease heterogeneity seems to have an unremarkable impact on VX-950 suppressive activity. Hence, it has been suggested that the majority of genotype-specific polymorphic sequences are located peripherally to the active sites of HCV protease, and do not affect binding of the agent molecule[26]. This investigation confirms the necessity for further research in this subject area. By contrast to the above are observations of telaprevir activity in a liver biopsy model of HCV infection. Cell cultures from liver biopsies of patients with genotype 1 and non-genotype 1 HCV were exposed to VX-950, which resulted in a significant decrease in HCV genotype 1 RNA, but only a minimal effect in non-genotype 1 HCV[27]. Thus further studies are required.

Another HCV protease inhibitor, SCH 503034, orally bioavailable with satisfactory pharmacokinetics and a good safety profile, is being tested in a phase Ⅱ clinical trial[28]. *In vitro* studies have determined its anti-viral activity on its

own, and an additive effect in combination with interferon α-2b[29,30]. Monotherapy with SCH 503034, at a dose of 400 mg q8h, in HCV-genotype-1-infected non-responders to prior standard therapy resulted in a 2.06 log reduction of the mean maximum viral load during the 14 d period of observation. Moreover, the HCV RNA decline correlated with ALT activity, and both of these parameters appeared to be dose-dependent. It is important to mention that the frequency of adverse events was comparable to the control group receiving a placebo[31]. Similar to telaprevir, further research into combination therapy with SCH 503034 and peginterferon has shown advantages over monotherapy with SCH 503034 or peginterferon. The mean maximum HCV RNA decline was dose-dependent, 2.4 log and 2.9 log for 200 mg q8h and 400 mg q8h, respectively, of SCH 503034 with peginterferon[32]. Another study designed to evaluate the antiviral effects of a combination of an NS3 protease inhibitor with a polymerase inhibitor strongly supports the concept of STAT-C. In this research, genotype 1b HCV replicon cells, wild-type, as well as replicons with reduced susceptibility to protease inhibitors or polymerase inhibitors, were exposed to a combination of SCH 503034 with the polymerase inhibitor HCV-796. The results were encouraging; the antiviral effect of these two agents was additive, with a favorable cross-resistance profile. Furthermore, the emergence of resistant viral variants was less frequent compared to that for monotherapy[33].

ITMN-191, a novel agent with a high genetic barrier to emergence of resistance mutations, is in preclinical development and has been shown to retain significant potency against variants with decreased sensitivity to other protease inhibitors[34]. Other *in vitro* studies have shown that ITMN-191 displays synergistic antiviral activity in combination with interferon[35,36], as well as HCV polymerase inhibitor R1479[37].

Recently, the clinical trials for BILB2061, another specific and potent inhibitor of HCV NS3 protease, were halted due to drug-induced cardiotoxicity[38].

ACH-806 is a novel anti-HCV agent, currently in phase Ⅰ/Ⅱ clinical studies, which has a distinct mechanism of action. Preclinical data have demonstrated that ACH-806 inhibits HCV genotype 1 replication *via* binding to NS4A and consequently blocks the formation of HCV replication complexes[39]. Resistance induction studies in replicon cells have shown a lack of cross resistance between ACH-806 and protease or polymerase inhibitors; thus confirming its different mode of action. Interestingly, viral quasispecies resistant to protease or polymerase inhibitors remain sensitive to ACH-806 and vice versa[40]. *In vitro* studies of a combination of ACH-806 with telaprevir or valopicitabine or interferon have demonstrated potent antiviral effects, which are synergistic when cells are exposed to ACH-806 with telaprevir or ACH-806 with valopicitabine. Hence the authors have concluded that ACH-806 could in the future become a potent component of STAT-C. Clinical studies preformed on genotype 1 HCV-infected subjects have shown an ~ 1 log reduction in HCV RNA at d 5. Further research on ACH-806 has been limited because of reversible nephrotoxicity reported in a clinical trial[41].

POLYMERASE INHIBITORS

The inhibitors of HCV NS5B polymerase influence different steps in RNA synthesis, from initiation to elongation through binding to the enzyme's active sites. Valopicitabine (NM283) appears the most advanced orally bioavailable pro-drug of a ribonucleoside analogue that displays an ability to sufficiently inhibit HCV NS5B polymerase. It is currently in phase Ⅱ clinical development. It has been shown that NM283 given as monotherapy exhibits significant, dose-related antiviral activity in both treatment-naïve and non-responders to prior interferon therapy. This effect was marked and synergistic, with a mean HCV RNA reduction of 2.7 log at d 28 when NM283 was administered in combination with peginterferon[42]. Studies in genotype 1 HCV-infected treatment-naïve patients showed an intense HCV RNA decline of about 4 log at wk 24 when NM283 was included in the regimen[43]. However evaluation of the side effects, mainly gastrointestinal and hematological disorders at higher doses, resulted in revision of the dosages. Interestingly, at higher doses, NM283 developed superior rapid response rates, although after 12, 24 and 36 wk of treatment the results were similar in all the different dose groups. Hence, low-dose NM283 in combination with peginterferon seems to display marked antiviral effects with fewer adverse events[43,44]. Moreover, there are promising results of *in vitro* research into the efficacy of NM283 against HCV variants resistant to protease inhibitors. All mutations tested conferring resistance to protease inhibitors were sensitive to NM283, thus supporting the advantages of combination therapy. As well, only a single mutation (S282T), described in the highly conserved B domain of NS5B, has so far been identified as determining moderate resistance to NM283[45]. In a separate study, a combination of two agents with distinct mechanisms of action, NM283 and SCH 503034, revealed enhanced anti-replicon activity, without observed cross-resistance[46]. These data support the initiative to develop combination regimens for treatment of HCV infections.

Other polymerase inhibitors currently in clinical development are nucleoside analogue R1479 (prodrug R1626) and non-nucleoside analogues HCV-796 and BILB1941. In phase I trials, R1626 has been shown to induce the greatest viral load reduction in genotype 1 HCV-infected patients, so far reported among this class of antiviral agents. It was a 3.7 log mean HCV RNA decline for 4500 mg q12h at d 14, and a 2.6 log decline for the lower dose of 3000 mg q12h, but with better tolerability[47]. *In vitro* analysis of the antiviral effects exerted by combinations of R1479 with interferon or ribavirin have demonstrated synergistic effects, whereas with other direct antivirals the effects were additive[48]. Another compound, HCV-796, when utilized in monotherapy exhibited dose-related anti-HCV activity across multiple genotypes, and with a good tolerability profile. Although, a selection of variants with reduced susceptibility was observed, they retained sensitivity to

interferon[49]. Associated studies of combined HCV-796 and peginterferon have confirmed the increased antiviral response to therapy, which was more intense in non-genotype 1 HCV[50]. In a clinical evaluation, polymerase inhibitor BILB 1941 showed anti-HCV activity; however, a high discontinuation rate due to adverse events within the group with appropriate pharmacokinetics was observed[51].

Several novel compounds are now being tested in preclinical trials; these include PSI-6130, GSK 625433, A-848837, A-837093 and AG-021541[52-56]. Additional studies should determine whether these polymerase inhibitors reach a clinical development stage in the future.

CYCLOPHILIN INHIBITORS

The favorable complementary elements of various prospective therapies seem to be the cyclophilin inhibitors, which exhibit peculiar mechanisms of action based on targeting of virus-host interactions. Cyclophilin B (Cyp B) has been shown to serve as a cellular cofactor of the NS5B RNA-dependent RNA polymerase that promotes HCV replication[57,58]. Thus the blocking of Cyp B appears to be an attractive target for the development of new anti-HCV agents[59-61]. The first compound shown to exhibit inhibitory properties with reference to cyclophilins was cyclosporin A (CsA)[60-62]. It was shown that its antiviral activity against HCV was distinct from its immunosuppressive function, which was dependent on anti-calcineurin activity[60-63]. Clinical studies on a regimen based on a combination of CsA with interferon-α demonstrated a high antiviral potency compared to that with interferon alone[63]. However, the immunosuppressive function of CsA limits its usefulness in anti-HCV therapy. Moreover, there are various opinions regarding the benefits of administering CsA *vs* tacrolimus in post-liver transplantation management of individuals with HCV infection[64,65]. The available data are limited, and the results are equivocal[66,67]. Thus, further studies are required in this area.

The first synthesized CsA derivate found to be devoid of immunosuppressive activity was NIM811. It has been investigated in regard to its anti-HIV inhibitory features[68,69]. Recent studies have confirmed its anti-HCV activity *in vitro*. The reduction of HCV RNA in replicon cells was more potent, even at low concentrations, compared to CsA. Moreover, NIM811 shows activity against HCV in replicons with high resistance to protease or polymerase inhibitors. Also, combinations of NIM811 with other groups of HCV inhibitors display at least additive effects[71]. Analogous results, with potentially synergistic effects, have been obtained when HCV replicon cells were exposed to a combination of NIM811 with interferon-α[72]. The anti-HCV activity of NIM-811 exerted at low drug concentrations infers reduced potential side effects from the therapy. Hence, *in vivo* evaluations of the safety profile, pharmacokinetics and anti-HCV activity need to be undertaken.

The first oral non-immunosuppressive cyclophilin B inhibitor, Debio 025, to enter into clinical trials displayed potent antiviral effects in chimeric mice, and the effect was enhanced in combination with pegylated interferon[72]. *In vitro* studies have demonstrated that Debio 025 has a unique potency for clearing HCV replicon cells of virus when used alone or in combination with other antivirals. The combination of Debio 025 with interferon α-2a results in additive to slightly synergistic antiviral effects. Resistant replicon cells, selected by passage in increasing concentrations of Debio 025, are sensitive to protease or polymerase inhibitors and interferon[73]. Moreover, Debio 025 exerts antiviral activity against wild-type HCV, as well as HCV replicons resistant to protease or polymerase inhibitors. The emergence of resistant variants to protease inhibitors (VX-950, BILB 2061) is restrained by the presence of Debio 025[74]. Clinical research shows that Debio 025 exerts both anti-HIV and anti-HCV activity in HIV/HCV co-infected subjects. The anti-HIV effect at d 15 was a 1 log decrease in viral load when the dose was 1200 mg q12h. HCV viremia decline was more profound, reaching 3.6 log at d 15. However, at this dose, transitional hyperbilirubinemia led to the discontinuation of treatment in some patients[6]. Debio 025 has been shown to exert anti-HCV activity in both genotype 1 and non-genotype 1 infections. Interestingly, no signals of emerging mutation appeared during the period of observation, which suggests a possible ability for this agent to suppress selection of resistant variants[75].

Recently the novel non-immunosuppressive CsA derivate, SCY-635, has been evaluated in preclinical studies that have demonstrated its favorable properties as an anti-HCV agent. Compared to CsA, this compound has good affinity with cyclophilin, and exerts potent antiviral activity with suitable pharmacokinetics and lower cytotoxicity. The combination of SCY-635 with interferon-α has antiviral effects on HCV replicon cells that range form additive to synergistic; thus supporting the need for more evaluations of this drug in anti-HCV treatment options[7].

CONCLUSION

Specifically targeted antiviral therapy, through interference in the replication cycle, leads to the selection of resistant variants[76-78]. This observation has been confirmed in several studies focusing on STAT-C agents as used in monotherapy. Combination therapy displays higher antiviral efficacy and has a fundamental impact on the selection of HCV strains with concomitantly reduced sensitivity and decreased viral fitness. It has also been shown that protease and polymerase inhibitors demonstrate advantageous cross-resistance profiles with improved anti-HCV activity, which supports the STAT-C development initiatives. Interestingly, the introduction of interferon to regimens based on novel compounds not only augments antiviral effects, but has a strong suppressive activity against the emergence of resistant mutations. Considering this, it is seems unlikely that interferon will be eliminated from antiviral regimens in the near future.

STAT-C presents an attractive strategy, one in which the main goal is to increase the effectiveness of antiviral responses across all genotypes, with shorter

Table 1 Anti-HCV agents currently under clinical development

Compound		Clinical advancement nt	Viral efficacy	Resistance (HCV resistant variants)	Remarks
Protease inhibitors	Telaprevir	Ⅱ	(dose-750 mg q8h; genotype-1)	Low level: V36A/M, T54A, R155K/T, A156S	Rash, GI and hematological adverse events (AE)
	(VX-950)	PROVE-1, 2, 3	VLR = 3 log d 3 VLR = 4.4 log: d 14 VLR = 5.5 log: d 14 when combined with PEG-IFNa2a	High level: A156V/T, V36A/M-R155K/T, V36A/M-A156V/T; After 14 d of treatment	
	SCH 503034	Ⅱ	(dose 400 mg q8h; genotype-1) VLR = 2.06 log d 14 VLR = 2.9 log: when combined with PEG-IFN	Low to moderate levels: V170A T54A A156S High level: A156T	Frequency of AE comparable to control group receiving placebo
Inhibitor of protease cofactor NS4A	ACH-806	Ⅰ / Ⅱ	VLR = 1 log: d 5	Single mutation at N-terminus of NS3; lack of cross resistance to any of the polymerase inhibitors or protease inhibitors now under development	Reversible nephrotoxicity
Polymerase inhibitors	Valopicitabine (NM283)	Ⅱ	VLR = 0.8 log: d 28 VLR = 2.7 log: d 28, when combined with PEG-IFN VLR = 4.24 log: wk 24, when combined with PEG-IFN	S282T	GI and hematological AE
	R1479 (R1626)	Ⅱ	VLR = 3.7 log for 4500 mg q12h at d 14, VLR = 2.6 log for 3000 mg q12h	no data	Mild to moderate hematological AE with increasing doses
	HCV-796	Ⅱ	VLR = 1.4-1.5 log: d 4 VLR = 3.3-3.5 log: d 14, when combined with PEG-IFN	C316Y	Mild to moderate headache-the most frequent AE; no treatment-emergent serious AE
	BILB 1941	Ⅰ	Data incomplete due to high discontinuation rate	No data	GI AE
Cyclophilin inhibitors	DEBIO 025	Ⅰ	VLR = 3.6 log: d 14 of monotherapy	No breakthrough during the treatment	Transitional hyperbilirubinemia

treatment duration and better tolerability. Recent studies have shown that these aims are within reach. Novel therapeutic agents are already in advanced clinical development; listed here in Table 1. Although there are many limitations pertaining to the STAT-C strategy; including variable bioavailability, and different effects exerted against various HCV genotypes and even quasispecies. A disadvantage of direct interference in the virus replication cycle is the increased rate of resistant mutations, but this could be ameliorated by the use of multiply agents with different mechanisms of actions. In combination with interferon, these drugs could augment antiviral effects and reduce some of the toxic side effects of this cytokine. On the other hand, the toxicity of new agents needs to be carefully evaluated, along with their pharmacokinetics, safety and effective doses. New regimens will also need to be worked out. Currently, it appears that interferon will remain as an element of anti-HCV therapy in the near future; indeed its effectiveness could be elevated by the introduction of new drugs.

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EDITORIAL

Persistent occult hepatitis B virus infection: Experimental findings and clinical implications

Patricia M Mulrooney-Cousins, Tomasz I Michalak

Patricia M Mulrooney-Cousins, Tomasz I Michalak, Molecular Virology and Hepatology Research, Division of BioMedical Science, Faculty of Medicine, Health Sciences Centre, Memorial University, St. John's, NL A1B 3V6, Canada
Supported by operating research grants from the Canadian Institutes of Health Research, Canada and the Canada Research Chair Program, and the Canada Foundation for Innovation
Correspondence to: Tomasz I Michalak, MD, PhD, Molecular Virology and Hepatology Research, Faculty of Medicine, Health Science Centre, Memorial University, 300 Prince Philip Drive, St. John's, NL A1B 3V6, Canada. timich@mun.ca
Telephone: +1-709-7777301 Fax: +1-709-7778279
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Abstract

Hepatitis B virus (HBV) is a highly pathogenic virus that causes chronic liver diseases in millions of people globally. In addition to a symptomatic, serologically evident infection, occult persistent HBV carriage has been identified since nucleic acid amplification assays of enhanced sensitivity became introduced for detection of hepadnaviral genomes and their replicative intermediates. Current evidence indicates that occult HBV infection is a common and long-term consequence of resolution of acute hepatitis B. This form of residual infection is termed as secondary occult infection (SOI). The data from the woodchuck model of HBV infection indicate that exposure to small amounts of hepadnavirus can also cause primary occult infection (POI) where virus genome, but no serological makers of exposure to virus, are detectable, and the liver may not be involved. However, virus replicates at low levels in the lymphatic system in both these forms. We briefly summarize the current understanding of the nature and characteristics of occult hepadnaviral persistence as well as of its documented and expected pathological consequences.

Key words: Hepatitis B virus; Hepadnaviruses; Woodchuck hepatitis virus; Hepadnaviral hepatitis; Occult viral persistence; Hepadnavirus lymphotropism; Primary occult infection; Secondary occult infection; Virus reactivation

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INTRODUCTION

Despite the availability of effective prophylactic vaccines, hepatitis caused by hepatitis B virus (HBV) remains one of the most troublesome infectious diseases responsible for significant morbidity and mortality in the world. HBV-induced chronic hepatitis B (CHB), defined as a prolonged liver inflammation accompanied by HBV surface antigen (HBsAg) in serum for longer than 6 mo, develops in up to 10% of individuals infected as adults and in about 85% of individuals exposed in the neonatal period and early childhood[1,2]. Consequently, up to 400 million people worldwide are afflicted with serum HBsAg-positive chronic infection, which in high endemic areas is mainly acquired by vertical virus transmission[3]. Furthermore, it is estimated that as much as one third of the world population have been exposed to HBV[4]. Many of these individuals may unknowingly carry the virus. It is assumed that, due to low virus loads in many of the cases, HBV may not be identifiable by commonly used serological assays based on detection of HBsAg and that the infection is often asymptomatic or has non-specific symptoms. This serologically and clinically unapparent infection makes the true global incidence of HBV hard to estimate. Consequently, the pathogenic significance of HBV might be much greater than that currently assumed.

OCCULT HBV INFECTION

The clinical data and the findings from animal models accumulated in the past ten years strongly argue that persistent, serologically silent, i.e., serum HBsAg-negative, HBV infection is a natural consequence of resolved acute hepatitis B, but it may also occur following an asymptomatic exposure to HBV, and may have serious pathological and epidemiological repercussions. Another important aspect of infections with HBV and other related viruses, that has been known for a long time but remained controversial and/or not fully realized, is the fact that the immune system, in addition to the liver, is the site of active hepadnaviral replication and long-term persistence.

Occult HBV infection is defined as the existence of HBV DNA in the serum, cells of the lymphatic (immune) system, and/or hepatic tissue in the absence of serum HBsAg. Most frequently, occult HBV infection follows

resolution of acute hepatitis and continues indefinitely after clearance of HBsAg and biochemical improvement in liver function[5-9]. For this reason, this form of infection is termed as residual or secondary occult infection or SOI, in opposite to primary occult infection or POI, which will be discussed later. Serological testing in SOI normally reveals the presence of antibodies to HBV core antigen (anti-HBc), which are now recognized not only as a valuable marker of prior exposure to HBV but also as an indicator suggesting actually progressing occult HBV infection. Nonetheless, recent estimates suggest that up to 20% of individuals with occult HBV carriage evidenced by HBV DNA detection could be nonreactive for anti-HBc or any other serological indicator of exposure to HBV[7]. It also is of note that the detection of naturally acquired antibodies to HBsAg (anti-HBs) does not exclude the existence of occult infection[5-7,9].

DETECTION OF OCCULT HEPADNAVIRAL PERSISTENCE

One of the main current challenges in diagnosis of occult HBV infection is the relatively low sensitivity of HBV DNA detection assays. The present assays are based on different approaches to amplification of HBV DNA by polymerase chain reaction (PCR) and their lowest detection limits range between 200 and 1000 virus genome copies, which are also referred to as virus genome equivalents (vge) per mL of plasma or serum. Although these assays are adequate to detect prodromal and symptomatic HBV infections and to monitor the progress of antiviral therapy, they are yet not sufficiently sensitive to identify occult infection in the majority of the individuals affected, where serum loads are usually below 100 vge per mL, as well as to determine whether virus was indeed completely eradicated following antiviral treatment[9]. In contrast to the clinical assays, the current research tests, which in our laboratory apply nested PCR and detection of resulting amplicons by nucleic acid hybridization (NAH), i.e., PCR/NAH, identify hepadnaviral DNA at loads below 10 vge/mL[10,11]. Furthermore, by testing of serial samples of serum and peripheral blood mononuclear cells (PBMC) and, when feasible, liver biopsies, and by amplifying different non-overlapping regions of the virus genome, detection of occult infection is greatly enhanced[9]. Application of these ultrasensitive assays to investigation of clinical materials and animal models, particularly the woodchuck model of hepatitis B, led to identification in the last decade of occult hepadnavirus infection as a new, distinctive entity and to delineation of its unique characteristics. However, due to yet limited population-based studies and relatively low sensitivity of the assays approved for clinical testing, the full impact of this infection remains to be determined. Nonetheless, with the recent introduction of clinical HBV nucleic acid testing (HBV NAT) in many parts of the world, significant progress in this area is expected.

EXPERIMENTAL OCCULT HBV INFECTION IN THE WOODCHUCK MODEL

Many of the characteristics of occult HBV infection have been originally identified in the woodchuck model of hepatitis B[5]. Woodchuck hepatitis virus (WHV) infecting the Eastern North American woodchuck (*Marmota monax*) is accepted as the most adequate, natural model of human HBV infection, hepatitis B and HBV-induced hepatocellular carcinoma (HCC). WHV closely resembles HBV in the structure, biology, and in the patterns and outcomes of the liver diseases induced. Much of the basic understanding of the natural course, requirements for transmission, molecular and immunological properties of occult hepadnaviral carriage, and its long-term pathological ramifications is owed to the woodchuck-WHV experimental system[10-15].

Occult WHV infection was first identified as a residual, life-long persistent infection in woodchucks who apparently completely resolved experimental acute hepatitis and developed anti-WHs (antibodies to WHV surface antigen, WHsAg)[10]. Analysis of sequential sera, PBMC and liver biopsies collected during the lifespan of these animals, as well as autopsy lymphatic and hepatic tissues, using sensitive PCR/NAH assays, identified WHV DNA in all three compartments, i.e., serum, lymphoid cells and the liver. In addition, the WHV replicating intermediates, covalently closed circular DNA (cccDNA), and virus mRNA were consistently found in the cells of the lymphatic system and the liver. Anti-WHc (equivalent of anti-HBc) persisted in all animals for life. This form of WHV infection become later termed as SOI[5,14]. In addition and unexpectedly, a low grade, intermittent liver inflammation was diagnosed throughout the remaining life in the majority of the woodchucks who apparently completely resolved acute hepatitis[10]. Not less importantly, one fifth of the animals developed HCC in 3-5 years after recovery and despite anti-WHs presence[10]. The same rate of HCC development was reported in woodchucks recovered from acute hepatitis which were examined by other investigators[16]. Most recently, it has also been shown that residual WHV in animals with SOI can be reactivated by treatment with an immunosuppressive agent, Cyclosporin A, leading to transiently serum WHsAg-positive infection[17]. Taken together, the data accumulated indicate that despite resolution of acute hepatitis, the replication of WHV continues for life, albeit at very low levels, both in the liver and the lymphatic system, that limited inflammatory process continues in hepatic tissue, and that the silently persisting virus may retain its oncogenic potency. This implies that although the immune system is able to resolve acute hepatitis and can keep persistently propagating hepadnavirus under relative control, it is unable to eradicate the virus completely[10,14]. The same conclusion was recently reached when bicistronic WHV core-gamma interferon (IFN) DNA vaccine was found to be able to prevent WHV hepatitis, but could not mount sterile immunity and prevent establishment of occult infection[18]. Furthermore, it became apparent that the detection of anti-WHc in the absence of other serological indicators of infection is a reliable indicator of occult WHV persistence[15]. This state, similarly as strong HBV-specific cytotoxic T cell (CTL) and T helper lymphocyte responses detectable years after resolution of acute hepatitis B[19,20], is likely a consequence of sustained restimulation of the immune system with a

viral protein produced during low-level virion assembly[15]. The high degree of compatibility between WHV and HBV infections and the data from studies on otherwise healthy anti-HBc-positive individuals[21,22] suggest that the occurrence of isolated anti-HBc could also be of value in identifying occult HBV persistence.

The virus recovered from woodchucks with SOI remains infectious. It was observed that WHV harvested from PBMC isolated during SOI and *ex vivo* stimulated with lipopolysaccharide (LPS) induced classical acute WHV hepatitis in virus-naive animals[10]. Furthermore, woodchuck dams with SOI were found to transmit virus to offspring, however, the infection induced frequently displays characteristics different than those of SOI[11]. Thus, WHV DNA and WHV cccDNA was harboured only in the lymphatic system, but not in the liver, and in the absence of serological markers of infection, including anti-WHc antibodies. This form of hepadnaviral infection was later named as primary occult infection or POI[14]. Interestingly, POI, in contrast to SOI, is not associated with protection from reinfection and hepatitis after challenge with liver pathogenic doses of WHV[11]. In subsequent studies, the same profile of occult infection was experimentally induced in adult animals by intravenous injections with WHV doses lower than 1000 virions[14]. At the same time, it was also established that WHV quantities higher than 1000 virions, in addition to infecting the lymphatic system, consistently cause classical acute hepatitis. This revealed that doses greater than 1000 virions are liver pathogenic in woodchucks[14]. From these data it was inferred that the threshold level of WHV required to infect lymphoid cells is 100 to 1000-fold lower than that to infect hepatocytes. WHV sequence variation was not responsible for the induction of these two contrasting forms of infection[14].

At the present time, POI is the entity which has not yet been convincingly identified in humans. However, the detection of occult HBV DNA-positive, anti-HBc-negative infection may suggest its existence. The persistence of trace amounts of replicating virus, especially at the extrahepatic locations, could have an impact in terms of unforeseen virus transmission and induction of disorders which are not yet considered to be related to hepadnaviral infection. Small quantities of the virus carried across the placenta may induce POI, similarly to CHB developing in neonates born to mothers with serum HBsAg-positive infection.

OCCULT HEPADNAVIRAL INFECTION AND THE HOST'S IMMUNE SYSTEM

Since the cells of the immune system are clearly the site of WHV propagation, independent of whether infection is symptomatic or occult, it became of interest to enumerate the cells carrying the virus in different forms of experimentally induced infection. By applying *in situ* PCR combined with flow cytometric quantification of cells containing amplified WHV core gene sequences, the proportion of WHV DNA-positive circulating lymphoid cells was found to range from 3.4% to 20.4% (mean 9.6%) in serum WHsAg-positive hepatitis, as compared to 1.1% to 14.6% (mean 4.8%) in occult WHV infection[13]. There was no difference between SOI and POI in the numbers of infected cells.

In terms of WHV-specific T cell responses, recent studies have shown that animals with POI display weak but evident, persistent and multispecific virus-specific T cell proliferative reactivity and that this cellular response does not provide protection against challenge with liver pathogenic doses of WHV[23] (Gujar *et al*, manuscript in preparation). The existence of active peripheral WHV-specific T cell response and innate immune cell activation in the livers of animals with SOI have also been established[12] (Gujar *et al*, manuscript in preparation). Overall, the findings in experimental SOI in woodchucks are closely compatible with those reported for humans convalescent from acute hepatitis B[19,20].

PRIMARY VERSUS SECONDARY OCCULT WHV INFECTION

In summary, experimental occult hepadnaviral infection occurs in two distinctive forms, as primary and secondary infection. POI is induced upon exposure to WHV doses at or below 1000 vge and engages the lymphatic system, although the infection with time may also spread to the liver[11,14]. Animals with POI carry virus in serum and lymphoid cells at levels which are comparable with those occurring in SOI and mount virus-specific T cell proliferative response, but they do not have serological (immunovirological) markers of infection and they are not protected from challenge with liver pathogenic doses of the virus. On the other hand, in SOI, a low-level replication of the virus progresses in both the liver and the lymphatic system, anti-WHc and frequently anti-WHs are detectable, and the animals are protected from hepatitis when re-exposed to liver pathogenic doses of WHV. Moreover, while SOI can be accompanied by low grade, protracted liver inflammation and HCC may finally develop, the liver is entirely normal in POI and a possible development of hepatoma has not yet been assessed.

IMPLICATIONS OF OCCULT HEPADNAVIRAL PERSISTENCE

Given the high degree of similarity between WHV and HBV and infections induced by these viruses, it is important to recognize the impact of silent HBV persistence in terms of its overall incidence, infectivity and pathogenic consequences. The previous clinical and research focus on the liver as essentially the only reservoir of HBV has somehow restricted characterization of extrahepatic sites of hepadnaviral replication and its potential pathological consequences. Recent studies are bringing occult HBV infection and the virus' lymphotropic nature to the forefront. Some of the cardinal works on occult HBV infection were accomplished by examining lymphoid cells of patients with resolved acute hepatitis B where HBV DNA sequences were found[19,24,25]. Furthermore, immunological studies showed that vigorous CTL and T cell proliferative

responses specific against HBV antigens persisted for years after recovery[19,20]. Many case reports also indicate that immunosuppression caused by chemotherapy[26,27], immunomodulatory agents[28], or immune deficiencies, such as HIV infection[29] or hematological malignancies[30], can reactivate occult infection. As in woodchucks with SOI, mild necroinflammation has been documented in liver samples obtained many years after recovery from acute hepatitis B[31,32]. Liver fibrosis and cirrhosis of unknown origin has now been explained by occult HBV infection in many retrospective studies[33,34]. There also is strong evidence for the risk of HCC development in patients with occult HBV[35-38] and this risk is further elevated in alcoholics[39] and in patients with other liver ailments, like hepatitis C[40]. Interestingly, convincing evidence points to the conservation of the HBV genome sequence in occult infection, negating the possibility that faulty HBsAg production accounts for all incidences of occult infection[32,41]. On the other hand, studies demonstrating the passage of HBV in non-liver cells, such as stem cells[42,43] and bone marrow[44], and by blood donations collected from individuals with occult HBV[45] are increasingly frequent in the literature. Recent estimates suggest that transient HBV DNA levels of up to 10^4 vge/mL can be found in sera from apparently healthy individuals with occult infection, which evade detection by current clinical assays, especially when testing is done on single serum or plasma samples[9,46].

CONCLUSION

The seriousness of the consequences of occult HBV infection is yet not fully recognized. Accumulated evidence indicate that occult HBV can be both a source of virus contamination in blood and organ donations, as well as the reservoir from which full blown hepatitis can arise. The oncogenic potency of occult HBV persistence becomes progressively evident. This silent infection can also affect the progression and outcomes of other viral diseases, particularly hepatitis C. It can be expected that while the burden of CHB will decrease with time due to introduction of more effective antiviral therapies, occult HBV infection could become a main concern. Understanding in its entirety of the nature and consequences of this form of HBV persistence should be of a high priority and this quest has already been initiated.

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REVIEW

Carbohydrate malabsorption in patients with non-specific abdominal complaints

Peter Born

Peter Born, Department of Internal Medicine, Tirschenreuth District Hospital, Tirschenreuth 95643, Germany
Correspondence to: Professor Peter Born, MD, Department of Internal Medicine, Tirschenreuth District Hospital, St. Peter-Strasse 31, Tirschenreuth 95643, Germany. peter.born@krankenhaus-tirschenreuth.de
Telephone: +49-9631-875204
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Abstract

Non-specific abdominal complaints are a considerable problem worldwide. Many patients are affected and many differential diagnoses have to be considered. Among these, carbohydrate malabsorption seems to play an important role. However, so far, only incomplete absorption of lactose is broadly accepted, while the malabsorption of fructose and sorbitol is still underestimated, although in many parts of the world it is much more frequent. Despite the success of dietary interventions in many patients, there are still a lot of unanswered questions that make further investigations necessary.

Key words: Non-specific abdominal disorders; Carbohydrate malabsorption; Fructose; Sorbitol; Lactose; Dietary intervention

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INTRODUCTION

The incomplete absorption of carbohydrates may cause serious problems for the affected patients, and is a frequent cause of so-called non-specific abdominal complaints. Non-specific abdominal complaints are characterized by a lack of morphological or biochemical abnormalities in the affected patients[1,2]. These complaints are a major problem throughout the world, with up to 30% of the population in Western countries being affected. Although only a minority seek medical attention, some 20%-50% of patients are referred to gastroenterologists so because of these non-specific complaints[2]. A study in Germany[3] has reported similar data, with 30% of the population being affected. A quarter of the patients required medical treatment, and no other diagnosis could be established in two-thirds of the cases. Similar findings have been described in Africa[4]; the rates appear to be lower in Asia, although they are still substantial, with a prevalence of up to 10% being reported[5].

There is a wide range of symptoms; the predominant ones are pain, flatulence, constipation and diarrhea. These symptoms should always be distinguished from those such as anemia, weight loss, bleeding and fever, which may indicate more dangerous conditions[6].

CARBOHYDRATE MALABSORPTION

Pathogenesis

Carbohydrates are a major source of calories in the diet. They may be ingested as monosaccharides, disaccharides, oligosaccharides or polysaccharides. During their passage from the mouth to the small intestine, they are broken down enzymatically until, in the small intestine, brush-border enzymes hydrolyze them into monosaccharides, which can be absorbed by various carrier systems[7].

The most important carbohydrates that routinely cause clinical abdominal complaints are lactose, fructose, and the sugar alcohol sorbitol. Lactose has long been recognized as one of the most important nutrients, and fructose and sorbitol have become increasingly important following recommendations to increase fruit and vegetable consumption, and also as a result of their use as sweeteners in dietary preparations and so-called sugar-free sweets. Further disorders such as sucrase-isomaltase deficiency or lack of trehalase are rare or only of regional importance, and are not discussed in this paper, although in affected patients, diets free from these sugars are very successful.

Malabsorption may result from congenital or acquired defects of single transport systems (primary malabsorption), or from impairment of the epithelial surface of the small intestine, due to general intestinal diseases such as celiac disease or Crohn's disease, which impede the absorption of all carbohydrates (secondary malabsorption). In secondary malabsorption, treatment is directed at the underlying disease, and successful therapy can lead to the normalization of carbohydrate absorption. In primary malabsorption, selective interventions are necessary.

Lactose is split by the enzyme lactase (β-d-

galactosidase), derived from the brush border of the enterocytes, into galactose and glucose, which can be absorbed by a special carrier system. The most frequently associated deficiency is primary lactase deficiency in adults. In these patients, lactase activity gradually declines during early life, and symptoms may occur as early as late childhood. Congenital lactase deficiency is rare and already affects neonates[8].

Several carriers, such as the GLUT family, are known to be involved in the transport of monosaccharides. GLUT-5 is the most important carrier currently known for the absorption of fructose. A deficiency in GLUT-5 leads to incomplete absorption or malabsorption of fructose[9,10]. Sorbitol is only sparsely absorbed, and is therefore used therapeutically as an osmotic laxative. Absorption is thought to take place through passive diffusion[11-13].

Congenital and acquired deficiencies are recognized. An important aspect is that the presence of glucose stimulates GLUT-5 activity, while sorbitol blocks it[9]. These data from animal intestinal perfusion tests (in most cases rat jejunum) confirm previous clinical and experimental data, which show that addition of glucose to fructose in patients known to have fructose malabsorption can prevent malabsorption, and suppress the occurrence of the accompanying symptoms. In contrast, the presence of sorbitol may aggravate malabsorption and the symptoms[13-17]. However, the mechanisms of carbohydrate absorption are still not completely understood.

There are also data that indicate an additional role for other factors in fructose metabolism, such as GLUT-2, which is capable of stimulating absorption[10].

Prevalence

Lactase deficiency is the most common enzymatic defect worldwide, although there are considerable regional differences in its prevalence. The majority of people in Asia and Africa, as well as the aboriginal populations of America and Australia, are affected, while in Europe, there are very low rates (under 10%) in the north, with a strong increase (up to 70%-100%) in regions further south, such as in Italy and Turkey.

There have been many studies reporting high malabsorption rates for fructose and sorbitol. Depending on the dosage and concentration, the reported data are closely comparable. After the ingestion of 50 g fructose dissolved in 250 mL water (corresponding to the traditional lactose test), there are malabsorption rates of about 60%-70%[15,18,19], while the rates are about 40% after a dosage of 25 g in 250 mL, the concentration now most commonly used[14,20]. Similar values have also been documented in children[21,22]. With regard to sorbitol, the test dosages used are less standardized, but even after an intake of 10 g, malabsorption rates can reach 100%[12,19,23].

For all of these sugars, it should be noted that the malabsorption rates are quite similar in patients and healthy controls. While some 50% of those with malabsorption of fructose and sorbitol have no concomitant symptoms, the symptomatic rates for lactose malabsorption are much more varied, with only a few people being symptomatic in some populations, despite high malabsorption rates.

Symptoms

The clinical symptoms of carbohydrate malabsorption include flatulence, abdominal cramps and pain, diarrhea, and sometimes even headache, usually after the ingestion of a product containing the incompletely absorbed sugar. There are no symptoms specific for a single sugar. However, there are data showing that, in patients (mainly females) with fructose and sorbitol malabsorption, diet can improve not only gastrointestinal disturbances, but also mood[24]. An association with reduced plasma levels of tryptophan has been discussed as a possible mechanism for this[25].

The gastrointestinal symptoms are thought to be provoked by the increased osmotic load of the sugar, with an augmented intraluminal volume (water) and a consequent acceleration of intestinal passage. Gas production and diarrhea occur in connection with the bacterial flora in the colon, the unabsorbed sugar presenting as a substrate for increased bacterial fermentation. Moreover, the capacity of the colon to absorb the surplus water plays a role in the pathogenesis of diarrhea.

In view of these etiologic factors, it is of particular interest that many patients report that initial symptoms develop after an infection (mostly gastrointestinal) or after antibiotic therapy, although it must be assumed that malabsorption has been present since childhood. Irritable bowel syndrome (which probably often includes patients with carbohydrate malabsorption) has also been reported to develop in connection with infections, both inside and outside the gastrointestinal tract[26,27]. In two small series, our own group has shown that the degradation of the malabsorbed sugar in stool cultures correlates with the occurrence of symptoms and a different pattern of short-chain free fatty acids[28,29].

It may therefore be speculated that the symptoms of carbohydrate malabsorption depend on the pattern of the colonic flora. A further argument in favor of this view is the observation that, in volunteers in whom more than one type of malabsorption is detected, the symptoms occur either after each malabsorption or after none[30].

Diagnosis

Carbohydrate malabsorption can be detected using direct or indirect methods. Using a cecal tube to measure the amount of unabsorbed sugar after oral ingestion is not practicable for routine purposes. Measurement of enzymatic activity in intestinal biopsies is a valuable tool for quantifying disaccharidase activity. It is therefore suitable for diagnosing lactase insufficiency, but is rarely used. Indirect methods are more widely used. Measurement of blood sugar after lactose ingestion was formerly a widespread method, but it declined in importance with the introduction of hydrogen exhalation tests. Direct measurement of ingested sugar in blood or urine is applicable for xylose.

By far the most frequently used method nowadays is the hydrogen exhalation test. After ingestion of the test sugar, the amount of hydrogen in the exhaled gas is measured. If there is incomplete absorption, part of

the ingested sugar passes into the colon, in which it is metabolized by bacteria into hydrogen, methane, carbon dioxide and free fatty acids. A small amount of hydrogen is absorbed and exhaled during the first passage through the lungs. An increase in hydrogen of more than 20 parts per million is considered to indicate malabsorption[31-33]. The extent of the hydrogen increase does not correlate either with the patients' symptoms or with the degree of malabsorption[34]. Hydrogen tests are only able to detect or exclude malabsorption. It should be pointed out that to avoid false-negative results, the presence of hydrogen-producing bacteria should be confirmed by carrying out a lactulose test (as lactulose is a disaccharide that cannot be absorbed by humans and therefore causes an increase in hydrogen). False-positive results due to bacterial overgrowth or accelerated orocecal transit can also be reduced with this test[35].

Concomitant measurement of blood glucose may improve the accuracy of lactose tests and should be mandatory in all fructose tests, in order to detect rare cases of fructose intolerance, which may mimic the symptoms of fructose malabsorption, but can cause severe hypoglycemia and even death after the ingestion of fructose. There is also some evidence that parallel methane assessment may improve the accuracy of the hydrogen test, but the data are sparse and require further evaluation[36]. The use of ^{13}C exhalation tests also requires further evaluation[37].

Therapy

Avoidance of the malabsorbed sugar is still the treatment of choice. In patients with lactose malabsorption, the addition of lactase (most conveniently in liquids) is an alternative, as well as the consumption of lactose-free milk products. Patients with fructose malabsorption can choose fruits and vegetables that have an equal ratio of fructose and glucose. In liquids, the addition of glucose can prevent malabsorption and its symptoms, but does not appear appropriate from the point of view of healthy nutrition. For sorbitol malabsorption, restricting sorbitol intake is the best method of preventing symptoms. In the same way that glucose can reduce malabsorption when administered along with fructose, adding sorbitol to fructose may aggravate the symptoms, and this should be taken into account when establishing the dietary regimen. For all sugars, small amounts are tolerated by most patients. Ingesting them along with or after other nutrients further improves tolerance.

The diet and its effects are often an important method of establishing a diagnosis of clinically relevant carbohydrate malabsorption. As mentioned above, many people are affected by malabsorption, but by no means are all symptomatic. Improvement after dietary adjustment and recurrence of symptoms when dietary errors occur is still the best way of confirming the diagnosis. There have been few studies that have documented the beneficial effects of dietary changes in patients with fructose and sorbitol malabsorption. In addition, there are methodological problems in all of these studies, including those by our own group. The diet itself is sometimes not clearly described; patient compliance with the diet is difficult to assess; there are no control groups, so that the role of a placebo effect is difficult to assess; and there are inadequacies in the way in which the patients' dietary behavior is recorded.

Nevertheless, the available data are fairly consistent. In our own study, which had both prospective and retrospective parts (in order to assess the placebo effect), symptomatic improvement of 60%-100%, depending on the degree of patient compliance, was observed to a similar degree in both arms of the study[38]. Fernandez-Banares et al[39] have reported similar results and were able to show that the treatment effect was long-lasting, with the positive effects of the diet being maintained at 12 mo. Shepherd and Gibson have confirmed these data in a group of 62 patients[40]. The largest study (so far available only as an abstract, so far as we are aware), including 1320 patients, documented an improvement after dietary changes in 87.5% of the patients[41]. In a more recent study including 90 selected patients with non-specific abdominal complaints, we observed a high rate of carbohydrate malabsorption (lactose 34%, fructose 61%, and sorbitol 91%)[42]. After dietary information had been provided, the patients reported significant improvement in 75% of cases, depending on the extent of compliance. The study by Ledochowski *et al*[24], which has shown that dietary changes in patients with fructose malabsorption have a beneficial effect on gastrointestinal complaints and also on mood, as documented by improvements in depression score, has already been mentioned above. However, there are also contradictory data that show that only avoiding one sugar (with proven malabsorption) may not be sufficient for patients with irritable bowel syndrome[43,44].

The phenomenon that patients with non-specific abdominal complaints frequently report improvement in their symptoms for several weeks after colonoscopy (thought to be due to bowel preparation with cleansing of the colon) underlines the possible role of bacteria in the colon. Treatments involving antibiotics[45,46], attempts to reduce colonic bacterial fermentation[47], and administration of probiotics[48] therefore require further investigation.

Open questions

Although carbohydrate malabsorption appears to play an important role in patients suffering from non-specific abdominal complaints, there are several problems with this diagnosis that should not be overlooked. In the first place, diagnosis using hydrogen tests is still problematic, particularly if the increase is borderline. The threshold value of 20 p.p.m. requires further evaluation, as there may be significant variations in methane production in the colon, among other factors. Moreover, hydrogen tests are not capable of quantifying the amount of malabsorption. Symptoms do not always occur during the test, even in patients who are considered to be symptomatic. Assessing the clinical relevance of the diagnosis may therefore sometimes be difficult.

The etiology of the symptoms is not fully understood. If the data published by our own group, which show that symptoms occur after each malabsorbed sugar or after none (see above), are reproducible, it would theoretically mean that no patient should become symptom-free, since

physiological malabsorption of carbohydrates is essential for the colonocytes. Moreover, if the data showing that there is a beneficial effect of carbohydrate malabsorption on the short-chain free fatty acid ratio, and especially on the increase in butyrate, are confirmed[29], this might also raise doubts about whether treatment is advantageous at all in the longer term.

CONCLUSION

Carbohydrate malabsorption is a common phenomenon not only in patients, but also in healthy individuals. In patients suffering from non-specific abdominal complaints, it is therefore very difficult sometimes to clarify whether the malabsorption that has been detected is definitely the cause of the symptoms. On the other hand, many patients with these symptoms and confirmed malabsorption appear to benefit considerably from dietary interventions, thus underlining the importance of diagnosing carbohydrate malabsorption. Another advantage of diagnostic work-up, including hydrogen exhalation tests, is that the results may provide evidence of other diseases that feature in the differential diagnosis of non-specific abdominal complaints, such as intestinal bacterial overgrowth or alterations in the orocecal transit times.

As pointed out above, however, there are still a considerable number of open questions in this field, so that further controlled studies are urgently necessary. The first step for the scientific community is to accept that, in addition to the widely accepted condition of lactose malabsorption, malabsorption of other sugars such as fructose and sorbitol also occurs and may be at least as important, or perhaps even more, than lactose malabsorption.

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GASTRIC CANCER

Metastatic suppressor genes inactivated by aberrant methylation in gastric cancer

Jian-Feng Wang, Dong-Qiu Dai

Jian-Feng Wang, Department of Surgical Oncology, the First Affiliated Hospital, China Medical University, Dalian University Affiliated Xinhua Hospital, Shenyang 110001, Liaoning Province, China
Dong-Qiu Dai, Department of Surgical Oncology, the First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China
Supported by the National Natural Science Foundation of China, No.30271477 and No.30572162; and the Special Scientific Research Foundation for Doctors, State Education Ministry, No.20050159001
Correspondence to: Dong-Qiu Dai, Department of Surgical Oncology, the First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China. daidq63@163.com
Telephone: +86-24-83283555
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Abstract

AIM: To screen out the differentially methylated DNA sequences between gastric primary tumor and metastatic lymph nodes, test the methylation difference of gene *PTPRG* between primary gastric tumor and metastatic lymph nodes, and test the regulatory function of 5-aza-2-deoxycytidine which is an agent with suppression on methylation and the level of methylation in gastric cancer cell line.

METHODS: Methylated DNA sequences in genome were enriched with methylated CpG islands amplification (MCA) to undergo representational difference analysis (RDA), with MCA production of metastatic lymph nodes as tester and that of primary tumor as driver. The obtained differentially methylated fragments were cloned and sequenced to acquire the base sequence, which was analyzed with bioinformatics. With methylation-specific PCR (MSP) and RT-PCR, methylation difference of gene *PTPRG* was detected between primary tumor and metastatic lymph nodes in 36 cases of gastric cancer. Methylation of gene *PTPRG* and its regulated expression were observed in gastric cancer cell line before and after being treated with methylation-suppressive agent.

RESULTS: Nineteen differentially methylated sequences were obtained and located at 5′ end, exons, introns and 3′ end, in which KL59 was observed to be located at 9p21 as the first exon of gene *p16* and KL22 to be located at promoter region of *PRPRG*. KL22, as the probes, was hybridized with driver, tester and 3-round RDA products respectively with all positive signals except with the driver. Significant difference was observed in both methylation rate of gene *PTPRG* and PTPRG mRNA expression rate between primary tumor and metastatic lymph nodes. Demethylation of gene *PTPRG*, with recovered expression of PTPRG mRNA, was observed after gastric cancer cell line being treated with methylation-suppressive agent.

CONCLUSION: Difference exists in DNA methylation between primary tumor and metastatic lymph nodes of gastric cancer, with MCA-RDA as one of the good analytical methods. Significant difference exists in methylation of gene *PTPRG* between primary tumor and metastatic lymph nodes of gastric cancer. Methylation level in gastric cancer cell line can be decreased by 5-aza-2′-deoxycytidine, which is the methylation-suppressive agent, with PTPRG expression being recovered.

Key words: Gastric cancer; Methylated CpG islands amplification; Representational difference analysis; DNA methylation; gene *PTPRG*

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http://www.wjgnet.com/1007-9327/13/5692.asp

INTRODUCTION

Metastasis of gastric cancer, at the genetic level, is caused by mutations or loss of corresponding cancer suppressor genes, while at epigenetic level it is caused by low expression of metastasis-suppression genes due to multiple reasons, in which gene hypermethylation is an important mechanism[1-3]. A genome with methylation at CpG is usually accompanied by inactivation of genes in that region. If not, on the contrary, it is usually accompanied by active expression of genes in the region. The aberrant hypermethylation of CpG islands in transcription regulatory region of metastasis-inhibition genes relating to gastric cancer causes these genes to be in silence and this fact will induce the metastasis of gastric cancer[4-6]. Most of previous analytic methods on methylation can only analyze the methylation condition of a known gene,

but cannot analyze that of a whole genome. Methylated CpG islands amplification (MCA) methods, combined with representative difference analysis (RDA), can analyze effectively the condition of methylation in a whole genome, especially good at detecting unknown methylated fragments.

In this research, gene methylation difference was detected between primary tumor and metastatic lymph nodes of gastric cancer using MCA-RDA method, to screen out genes relating to metastasis of gastric cancer, which further underwent analysis on methylation difference with methods, including methylation-specific PCR, in order to define further the mechanism of metastasis of gastric cancer.

MATERIALS AND METHODS

Subjects

Pathological specimens, including tumor tissues and metastatic lymph nodes, taken from 36 gastric cancer patients hospitalized in Surgical Oncology Ward of the First Affiliated Hospital of China Medical University, were included in this study. The tumor tissues and suspected metastatic lymph nodes, after resecting from the patients, were promptly placed into a liquid nitrogen tank. Each tumor tissue and lymph node was cut into two pieces, one piece was kept in liquid nitrogen and the other underwent HE pathological staining to examine whether a true metastasis had occurred. In addition, gastric cancer line SGC7901 was also included in this study.

Extraction of DNA and total RNA

Hydroxybenzene-chloroform extraction method was adopted to extract DNA of the genome, and total RNA was extracted with TRIZOL reagent according to the manufacturer's instruction.

MCA

MCA was adopted to obtain CpG islands enriched with methylation. The CpG island region of DNA mixture extracted from tumor tissues and metastatic lymph nodes of 5 cases of gastric cancer were enriched with MCA, respectively. Firstly, 5 μg of DNA of genome was digested with 100U *Sma*I endonuclease (provided by NEB, not functional on methylated sites) for 6 h to cut the unmethylated -CCCGGG- sites to form the blunt ends, and also digested with 20U *Xma*I enzyme (provided by NEB) for 16 h to cut the methylated -CCCGGG- sites to form the sticky -CCCGGG- ends. Then T4DNA (Promega) was used to connect corresponding adaptor RXMA 24/12, with RXMA24 fragment as the primer to amplify the DNA fragments with adaptor, which were incubated for 5 min at 72℃, followed by pre-denaturalization for 3 min at 95℃, 30 amplification cycles for 1 min at 95℃ and 3 min at 72℃, and a final extension for 10 min at 72℃, to enrich methylated fragments of DNA from both tumor tissues and metastatic lymph nodes. The products were electrophoresed on 15 g/L agarose gel containing ethidium bromide[7].

Table 1 Adaptor used in MCA-RDA and their sequence

Adaptor	Sequence
RXMA24	5'-AGCACTCTCCAGCCTCTCACCGAC-3'
RXMA12	5'-CCGGGTCGGTGA-3'
JXMA24	5'-ACCGACGTCGACTATCCATGAACC-3'
JXMA12	5'-CCGGGGTTCATG -3'
NMCA24	5'-GTTAGCGGACACAGGGCGGGTCAC-3'
NMCA12	5'-CCGGGTGACCCG-3'

RDA

The methylated DNA fragments obtained from tumor and metastatic lymph nodes were underwent representative difference analysis. The adaptor of methylated CpG fragments taken from tumor and metastatic lymph nodes was cut-off with *Sma*I being used for tumor tissue to form blunt ends as the driver, and with *Xma*I being used for metastatic lymph nodes to form sticky ends to be connected with new ends as the tester. Tester and driver were underwent 3 cycles of hybridization RDA analysis in a ratio of 1:80, 1:400 and 1:800, respectively. After each analysis, the adaptor was cut off with *Xma*I, with new adaptor being added. The adaptors used in the 3 cycles of analysis were NMCA24/12, JXMA24/12 and NMCA24/12, with different extension temperature for different connectors. The sequence of each connector is listed in Table 1. The products were analyzed on 15 g/L agarose gel containing ethidium bromide[8].

Cloning, sequencing and analysis of similarity

Products of the 3rd cycle of RDA analysis as well as pCAT®3-Control carrier (Promega) were treated with *Xma*I to cut their ends into sticky ones, which were connected with T4 ligase and transformed into competent bacteria JM109 for incubation with matrix containing Ampicillin. Positive clones were selected and cultivated in matrix containing antibiotics at 37℃. Then plasmid DNA was extracted, and was underwent to cleavage with *Xma*I, and to electrophoresis; then the more than 100 bp and the clones of more than 100 bp cleavage products were selected and delivered to bio-company (Combined Gene Company) for sequencing. The obtained sequence were underwent repeated sequence analysis with Repeatmasker. BLAST system was used to carry out similarity analysis, with relationship between cloned sequence and corresponding genes being analyzed *via* GenBank.

Dot blot

The differentially methylated fragments of KL22 obtained from MCA-RDA analysis were labeled with digoxin, using random primer method to form the probe. With this latter hybridization analysis was carried out on the 1st, 2nd, 3rd round RDA. MCA products of tumor or metastatic lymph nodes, respectively, in a volume of 5 μL for each sample, were dotted onto nylon membrane with positive electricity.

Cell cultivation and methylation intervention

Gastric cancer cell line was subcultured according to

Table 2 MSP primers of gene *PTPRG*

Methylated primers	5'-GTTCGTTCGTTTTTTCGTTC-3' 5'-CATACTCCTAAAAATTATAACTCCGAC-3'
Unmethylated primers	5'-TTTTGTTTGTTTGTTTTTTTGTTTG-3' 5'-AATCCATACTCCTAAAAATTATAACTCCA-3'

Table 3 RT-PCR primers of *PTPRG* gene

PTPRG primer	5'-CTAATAAGGGATGTTCACATGAAGC-3' 5'-CTGTATTTTAATGGAGTGGATAGCA-3'
β-actin primer	5'-AAATCGTGCGTGACATTAA-3' 5'-CTCGTCATACTCCTGCTTG-3'

standard methods and then randomized into two groups, one of them was treated with 5 μmol/L 5-Aza-2'-deoxycytidine and cultured for 5 d.

Methylation-specific PCR (MSP)

Sodium hydrogen sulfite was used for DNA modification, and then sodium hydrogen sulfite was eliminated from DNA with Wizard DNA Clean-up kit (Promega). The samples were amplified through 30 cycles, each amplification cycle consisting of denaturation at 95℃ for 40 s, primers annealing at 65℃ (unmethylation) or at 60℃ (methylation) for 40 s and extension at 72℃ for 60 s. Cycles were preceded by incubation at 95℃ for 3 min to ensure full denaturation of the target gene, and finally by an extra incubation at 72℃ for 10 min to ensure full extension of the products. PCR was carried out with methylated primer and unmethylated primer, respectively. The primers adopted are listed in Table 2. The PCR products were analyzed on 20 g/L agarose gel[9].

RT-PCR

RNA was reverse transcribed into cDNA as the template, which was amplified through 30 cycles, each amplification cycle consisting of denaturation at 94℃ for 40 s, primers annealing at 57℃ for 30 s and extension at 72℃ for 60 s. Cycles were preceded by incubation at 94℃ for 2 min to ensure full denaturation of the target gene, and finally by an extra incubation at 72℃ for 10 min to ensure full extension of the products.

Meanwhile, β-actin was adopted as internal control. The sequences of primers are listed in Table 3. The products were electrophoresed on 15 g/L agarose gel.

Statistical analysis

Chi-square test was adopted to verify the difference of PTPRG methylation rate and PTPRG mRNA expression between gastric tumor and metastatic lymph nodes, as well as the difference on absent expression of PTPRG mRNA between negative and positive group of methylated nodular PTPRG. Rectilinear regression was used to test the correlation between PTPRG methylation rate and metastatic lymph nodes number. SPSS11.0 software was used to process the data.

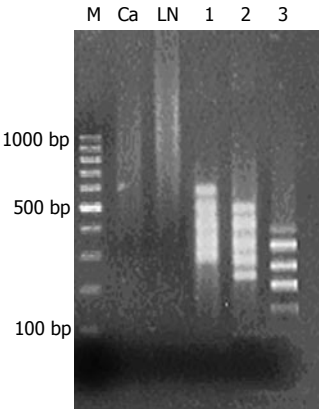

Figure 1 Methylated CpG islands amplification (MCA) and representational difference analysis (RDA). M: Marker; Ca: MCA products of gastric cancer tissues; LN: MCA products of metastatic lymph nodes; lanes 1-3: The 1st to the 3rd round RDA products. After methylated CpG islands amplification (MCA) of genome DNA of primary tumor and metastatic lymph nodes, bright smears were observed between 300 bp and 2000 bp, which were the concentrated methylated CpG islands. From the 1st to the 3rd cycle of analysis, fragments with methylation difference decreased gradually and the straps gradually became clear. In the 3rd RDA analysis, 5 straps of different methylation were observed.

RESULTS

MCA

After methylated CpG islands amplification (MCA) of genome DNA of primary tumor and metastatic lymph nodes, bright smear was observed between 300 and 2000 bp, which were the concentrated methylated CpG islands (Figure 1).

RDA

MCA products of metastatic lymph nodes were adopted as the tester and MCA products of primary tumor as the driver to carry out 3 cycles of RDA analysis, which resulted in 100-500-bp fragments with methylation difference. From the 1st to the 3rd cycle of analysis, fragments with methylation difference decreased gradually and the straps gradually became clear. In the 3rd RDA analysis, 5 straps of different methylation were observed (Figure 1).

Cloning, sequencing and analysis on homology

Ninety-six positive clones were selected to undergo sequencing analysis, 19 of them demonstrated the sequence longer than 100 bp. KL8 appeared for 21 times, while KL22 11 times, KL59 4 times, and both KL40 and KL71 for twice. No repeated sequence, such as ALU, was found after repeated sequencing analysis. Table 4 shows the 19 sequence characteristics. All sequences were of length between 100 bp and 400 bp, with GC content beyond 50%. Analysis showed these fragments to be distributed into various regions in the genome, including 5' ends, exons, introns and 3' ends, in which KL59 was situated at 9p21 as the first extron of gene *p16*, with 100% similarity rate, and KL22 was situated at 3p21, in the promoter region of gene *PTPRG*.

Dot blot

KL122 sequence was labeled with digoxin to form the

Table 4 Features of fragments with different methylation

Fragment	Length (bp)	GC %	CpG/GpC	Chromosome Positioning	Similarity rate %	S	E
KL2	198	55.0	0.5625	1q23.1	100	404	e-110
KL5	106	71.2	0.7142	15	100	222	e-56
KL6	159	59.4	1.0714	1p36.31-36.23	98	141	2e-31
KL8	194	61.1	0.8461	2q33.3	99	396	e-108
KL14	347	72.3	0.9811	4p15	100	585	e-165
KL19	258	58.3	0.7692	5p15.1	97	480	e-133
KL22	332	64.1	0.7027	3p21	99	527	e-147
KL23	287	70.7	1.0606	1q42.1-43	98	458	e-126
KL33	136	53.2	0.9629	2p24.3-24.1	100	129	6e-28
KL40	255	66.2	1.1428	4p16.1	99	404	e-110
KL55	268	65.6	0.7894	18	92	231	e-152
KL59	282	64.5	0.6471	9p21	100	571	e-160
KL68	251	62.5	0.6000	4	87	173	4e-39
KL71	213	69.9	0.8260	10p12.1	100	434	e-119
KL74	341	69.5	0.7317	9p21	99	668	0
KL79	225	61.3	0.4782	13q13	98	458	e-127
KL82	403	67.4	0.8043	8q21.2	99	383	e-104
KL87	275	51.9	1.0000	11	97	515	e-144
KL95	360	70.8	0.7608	Xp22.3	100	726	0

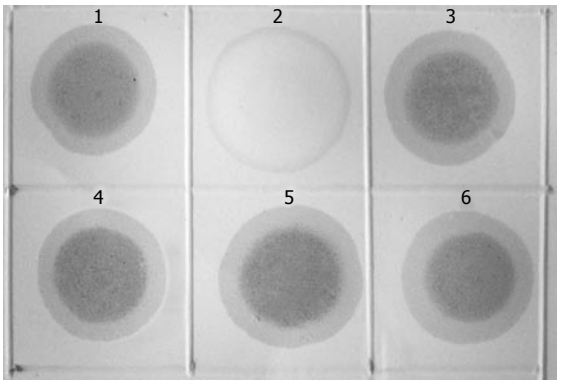

Figure 2 Dot blot analysis. 1: Positive control; 2: MCA products of gastric cancer tissues; 3: MCA products of metastatic lymph nodes; and 4-6: the first to the third round RDA products. KL122 sequence was labeled with digoxin to form the probe; with this latter the three rounds MCA-RDA products were undergone hybridization analysis with testers and drivers. Positive results were observed in all products of the three-round RDA as well as in testers, while negative one in drivers.

probe, with which the three rounds MCA-RDA products underwent hybridization analysis with testers and drivers. Positive results were observed in all products of the 3-round RDA as well as in testers, while negative one in drivers (Figure 2).

Gene PTPRG methylation rate of primary tumor and metastatic lymph nodes of gastric cancer

A positive band was observed at 158 bp of non-methylation PCR of primary tumor, with a positive rate of 77.78% (28/36), while that of metastatic lymph nodes was 63.89% (23/36) ($P > 0.05$). A positive strap was observed at 150 bp of methylation PCR of metastatic lymph nodes, with a positive rate of 52.78% (19/36), while that of primary tumor was 25.0% (9/36) ($P < 0.05$) (Figure 3 and Table 5). Linear correlation was observed between MSP positive rate of metastatic lymph nodes and the number of metastatic nodes ($r = 0.882$, $P < 0.001$, Figure 4).

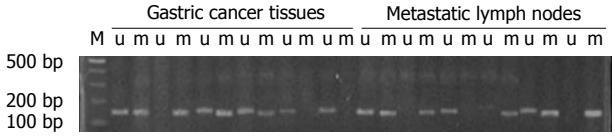

Figure 3 Methylation-specific PCR (MSP) of gene *PTPRG*. M: Marker; u: Unmethylated (158 bp); m: Methylated (150 bp). A positive band was observed at 158 bp of non-methylation PCR of primary tumor, with a positive rate of 77.78% (28/36), while that of metastatic lymph nodes was 63.89% (23/36). A positive strap was observed at 150 bp of methylation PCR of metastatic lymph nodes, with a positive rate of 52.78% (19/36), while that of primary tumor was 25.0% (9/36) ($P < 0.05$).

Table 5 Methylation and mRNA expression of gene *PTPRG n* (%)

Tissue	*PTPRG* gene methylation		PTPRG mRNA expression
	U	M	
Primary tumor	28 (77.78)	9 (25.0)	18 (50.0)
Metastatic lymph nodes	23 (63.89)	19 (52.78)[a]	9 (25.0)[a]
Before cell line treatment	+	+	-
After cell line treatment	+	-	+

[a]$P < 0.05$. U: Unmethylation; M: Methylation.

A positive strap was observed at 177 bp in RT-PCR of gene *PTPRG* of primary tumor, with a positive rate of 50.0% (18/36) and a 177-bp positive band was observed in metastatic lymph nodes, with a positive rate of 25.0% (9/36) ($P < 0.05$), (Figure 5, Table 5).

Relationship between methylation of PTPRG in metastatic lymph nodes and absent expression of mRNA

Among 19 cases of positive PTPRG methylation in metastatic lymph nodes, there was only one case of positive expression of PTPRG mRNA, with the positive rate of 5.26%, while 9 cases of positive expression existed among 17 cases of negative PTPRG methylation, with the positive rate of 52.9% ($P < 0.01$), (Table 6).

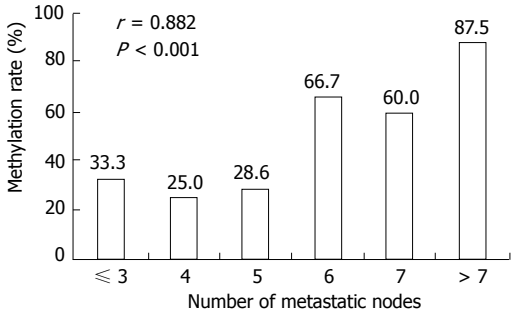

Figure 4 Relationship between number of metastatic lymph nodes and positive rate of PTPRG methylation.

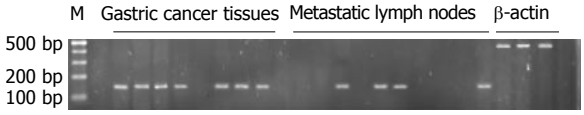

Figure 5 PTPRG mRNA expression of gastric cancer tissues and metastatic lymph nodes. M: Marker. Gastric cancer tissues: Products of *PTPRG* gene (177 bp) of gastric cancer tissues; Metastatic lymph nodes: Products of *PTPRG* gene (177 bp) of metastatic lymph nodes; β-actin: 483 bp. RT-PCR showed a positive strap at 177 bp in gene *PTPRG* of primary tumor, with a positive rate of 50.0% (18/36), while a 177-bp positive band was observed in metastatic lymph nodes, with a positive rate of 25.0% (9/36) ($P < 0.05$).

PTPRG methylation level and its mRNA expression in gastric cell line SGC7901 before and after the treatment with 5-aza-2'-deoxycytidine

Before the treatment, a positive band was observed at 158 bp in unmethylated PCR, while a positive band was seen at 150 bp in methylated PCR of gastric cancer cell line. After the treatment, a positive band was also observed at 158 bp in unmethylated PCR, but no positive band was seen in methylated PCR (Figure 6, Table 5). PTPRG mRNA expression of cell line was negative before treatment, while a weak positive band was seen at 177 bp after treatment (Figure 7, Table 5).

DISCUSSION

Based on previous researches, a group containing methyl exists in every 100 nucleotide acids in human DNA, which is usually combined onto 5'-C position. Almost all methylated cytosine residues appear on the 5'-GC-3' nucleotide acid in the symmetrical sequence. This kind of sequence is not randomly distributed, but concentrated to GC-enriched islands (CpG islands), which usually situates at the position in or near transcription regulatory region. Sensitivity of methylation on all CpG is not the same, and the methylation level at the site of CpG can be changed[10-12]. Prevalent hypomethylation and local hypermethylation exist in genome of cancer tissue, for example, hypermethylation on promoters of p16, E-cadherin, and genes encoding hormone receptors and genes of DNA repair, and genes inhibiting the genesis of blood vessels may induce the absent or low expression of these gene and improve the oncogenesis and metastasis. Therefore, research on methylation of genome provides a new route to study the oncogenesis and metastasis of cancer[13-17].

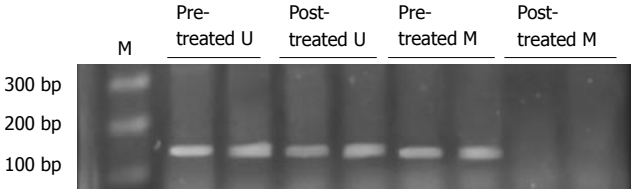

Figure 6 Methylation-specific PCR (MSP) of gene *PTPRG* in gastric cell line before and after the treatment with 5-aza-2'-deoxycytidine. M: Marker; U: Unmethylated; M: Methylated. Before the treatment, a positive band was observed at 158 bp in unmethylated PCR, while a positive band was seen at 150 bp in methylated PCR of gastric cancer cell line. After the treatment, a positive band was also observed at 158 bp in unmethylated PCR, but no positive band was seen in methylated PCR.

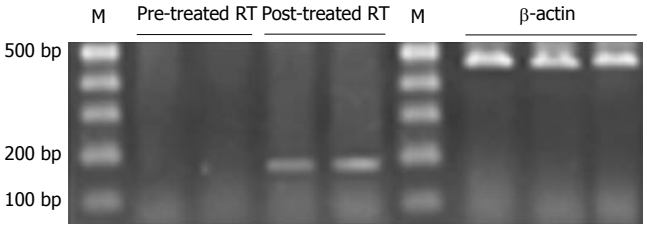

Figure 7 PTPRG mRNA expression of cell line before and after the treatment with 5-aza-2'-deoxycytidine. M: Marker; Pre-treated RT: PTPRG mRNA expression before the treatment with 5-aza-2'-deoxycytidine; Post-treated RT: PTPRG mRNA expression after the treatment with 5-aza-2'-deoxycytidine.

Table 6 The relationship between PTPRG methylation in metastatic lymph nodes and absent expression of mRNA.

PTPRG methylation	PTPRG mRNA expression (%)	
	+	-
+	1 (5.26)	18 (94.73)
-	9 (52.94)	8 (47.05)

$\chi^2 = 10.17$, $P < 0.01$.

MCA technique is a new approach which has recently been adopted for research on gene methylation, which can be applied promptly and efficiently to the research on the whole genome methylation, with specific advantage especially for research on methylation condition of various unknown genes. Through the optimization of PCR condition, it is almost fit for every gene containing two adjacent *Sma*I restriction sites. The content of CpG is different in CpG islands, so different PCR primers and reaction conditions are needed, such as RXMA and RMCA. Thus RXMA seems to be more fit for this research. Through the optimization on the reaction condition, RXMA may yield MCA product steadily. It needs to point out that high quality sample of DNA is needed for MCA experiment, so generally wax-embedded sample is not considered in the case, in which only some sites can be detected, but not in total, among all sites in the CpG islands, which is only sensitive to partially digested products by *Sma*I, with distance shorter than 1000

bp between every two *Sma*I constriction sites. Generally speaking, GC content of MCA's products is high; in this case if only an optimized PCR reaction system is adopted with high GC content, a satisfactory result can be made. RDA is a relatively mature technique adopted to screen out accurately the different fragments between two groups of DNA. In this research, we combined MCA and RDA to screen out the differentially methylated fragments between primary tumor and metastatic lymph nodes of gastric cancer, to explore the gene with alteration in methylation involved within process of metastatic lymph nodes. It provides a relatively accurate method with high efficiency, and it is fit for the primary screening out large quantities of metastasis-suppressive genes. It may be considered a high flux analytical method.

Phosphorylation of tyrosine residue is the important characteristic of many cellular signals transmission, influencing a number of vital biological processes including growth and differentiation of cells, adjustment of cell cycle, cell apoptosis and transference. Phosphorylation and dephosphorylation of tyrosine are adjusted by tyrosine kinase (TK) and tyrosine phosphatase (TP), respectively. Despite several tyrosine kinases have been recognized to correlate to oncogenesis directly through activating the mutant of cells *in vivo*, only a small amount of tyrosine phosphatase is known to correlate oncogenesis[18,19]. Gene *PTPRG* is a member belonging to the classic tyrosine phosphatase gene family, which includes receptor genes and non-receptor genes. *PTPRG* is a member of receptor gene, and is situated at 3p14 chromosome[20-22]. Previous researches observed methylation extinguishments of other genes that belong to the same family of *PTPRG*, such as *PTPRN2*, *PTPRO*, *etc*, in hepatic cancer[23]. *PTPRG* mutation is often found in colon cancer, lung cancer and kidney cancer, with conclusive identification on the role of *PTPRG* as one of tumor-inhibition genes[18,24]. However, there is a little information in literature regarding the extinguishments of *PTPRG* on epigenetic level. Based on recent studies, its deactivation on epigenetic level occurs in skin T-cell lymphoma. A study has demonstrated that a significant difference exists on *PTPRG* methylation in metastatic lymph nodes compared to primary tumor[9]. The silencing of genomic induced by methylation mainly consists of the methylation in promoter and in the first exon[9].

Methylation-specific PCR (MSP) revealed a significant difference in the positive methylation rate of PRPGR in primary tumor of gastric cancer (25.0%) compared to in metastatic lymph nodes (52.78%) ($P < 0.05$)[25,26]; this fact which further proved that the differentially methylated fragments we screened out were accurate. The significant linear correlation existing between positive rates of methylated *PTPRG* and numbers of metastatic nodes suggests that there is certain relationship between *PTPRG* methylation and metastatic lymph nodes of gastric cancer. In addition, a significant difference in *PTPRG* mRNA expression was observed between primary gastric tumor and metastatic lymph nodes, suggesting that product of *PTPRG* gene exerts suppressive effect against metastatic lymph nodes of gastric cancer. It is because of the down-regulated PTPRG, that the metastatic lymph nodes of gastric cancer was promoted.

PTPRG methylation exists in gastric cancer cell line. RT-PCR analysis demonstrated that PTPRG is not expressed in the cell line. However, MSP result of the cell line was negative and RT-PCR result was weakly positive after treatment with 5-aza-2'-deoxycytidine as the methylation suppressor; this fact, suggested that methylation of this gene was suppressed after the treatment with 5-aza-2'-deoxycytidine, so the MSP result was negative and the expression of PTPRG was partially recovered[27,28].

Among all 19 cases of positive methylation of PTPRG in metastatic lymph nodes, there was only one case of positive expression of PTPRG mRNA (positive rate of 5.26%), while among 17 cases of negative methylation of PTPRG, there were 9 cases of positive expression of PTPRG mRNA (positive rate of 52.9%) ($P < 0.05$); this result further implies that methylation in promoter region of PTPRG might be the mechanism of its being distinguished. However, the concrete mechanism of this gene involved in metastatic lymph nodes of gastric cancer is not clear yet, and it is waiting for the further research.

COMMENTS

Background

Metastasis of gastric cancer, at the genetic level, is caused by mutation or loss of corresponding cancer suppressor genes, while at epigenetic level it is caused by low expression of metastasis-suppression genes due to multiple reasons, among which gene hypermethylation is an important mechanism.

Research frontiers

Methylated CpG islands amplification (MCA) methods, combined with representative difference analysis (RDA), can analyze wholly and effectively the condition of methylation in a whole genome, especially good at detecting unknown methylated fragments.

Innovations and breakthroughs

In this research, gene methylation difference was detected between primary tumor and metastatic lymph nodes of gastric cancer using MCA-RDA method, to screen out genes relating to metastasis of gastric cancer, which further underwent analysis on methylation difference by methylation specific PCR, in order to define further the mechanism of metastasis of gastric cancer.

Applications

This observation might be of potential value in gene therapy of gastric cancer.

Peer review

The manuscript looked at the potential role of the *PTPRG* gene as metastatic suppressor gene, by comparing methylation status and LOI between the primary tumor and lymph nodes metastasis in 36 cases. Additionally, gastric cancer cell line was treated with 5-aza-2'-deoxycytidine (inhibitor of methylation) and gene expression was investigated. This study proved the hypothesis by demonstrating a significant difference in the level of methylation between the primary tumor and metastasis.

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Characteristics and pathological mechanism on magnetic resonance diffusion-weighted imaging after chemoembolization in rabbit liver VX-2 tumor model

You-Hong Yuan, En-Hua Xiao, Jian-Bin Liu, Zhong He, Ke Jin, Cong Ma, Jun Xiang, Jian-Hua Xiao, Wei-Jian Chen

You-Hong Yuan, Jian-Bin Liu, Department of Radiology, Hunan Province People's Hospital, Changsha 410005, Hunan Province, China
En-Hua Xiao, Zhong He, Cong Ma, Jun Xiang, Department of Radiology, the Second Xiang-Ya Hospital, Central South University, Changsha 410005, Hunan Province, China
Ke Jin, Wei-Jian Chen, Department of Pathology, Hunan Province Children's Hospital, Changsha 410005, Hunan Province, China
Jian-Hua Xiao, Department of Epidemiology, Center for Disease Control of Hunan Province, Changsha 410005, Hunan Province, China
Supported by the National Natural Science Foundation of China, No. 30070235, 30470508
Correspondence to: Dr. You-Hong Yuan, Department of Radiology, Hunan Province People's Hospital, Changsha 410005, Hunan Province, China. heyuanyouhong@yahoo.com.cn
Telephone: +86-731-2278047 Fax: +86-731-2278011
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Abstract

AIM: To investigate dynamic characteristics and pathological mechanism of signal in rabbit VX-2 tumor model on diffusion-weighted imaging (DWI) after chemoembolization.

METHODS: Forty New Zealand rabbits were included in the study and forty-seven rabbit VX-2 tumor models were raised by implanting directly and intrahepatically after abdominal cavity opened. Forty VX-2 tumor models from them were divided into four groups. DWI was performed periodically and respectively for each group after chemoembolization. All VX-2 tumor samples of each group were studied by pathology. The distinction of VX-2 tumors on DWI was assessed by their apparent diffusion coefficient (ADC) values. The statistical significance between different time groups, different area groups or different b-value groups was calculated by using SPSS12.0 software.

RESULTS: Under b-value of 100 s/mm^2, ADC values were lowest at 16 h after chemoembolization in area of VX-2 tumor periphery, central, and normal liver parenchyma around tumor, but turned to increase with further elongation of chemoembolization treatment. The distinction of ADC between different time groups was significant respectively (F = 7.325, P < 0.001; F = 2.496, P < 0.048; F = 6.856, P < 0.001). Cellular edema in the area of VX-2 tumor periphery or normal liver parenchyma around tumor, increased quickly in sixteen h after chemoembolization but, from the 16th h to the 48th h, cellular edema in the area of normal liver parenchyma around tumor decreased gradually and that in the area of VX-2 tumor periphery decreased lightly at, and then increased continually. After chemoembolization, Cellular necrosis in the area of VX-2 tumor periphery was more significantly high than that before chemoembolization. The areas of dead cells in VX-2 tumors manifested low signal and high ADC value, while the areas of viable cells manifested high signal and low ADC value.

CONCLUSION: DWI is able to detect and differentiate tumor necrotic areas from viable cellular areas before and after chemoembolization. ADC of normal liver parenchyma and VX-2 tumor are influenced by intracellular edema, tissue cellular death and microcirculation disturbance after chemoembolization.

Key words: Liver; VX-2 tumor; Diffusion-weighted imaging; Apparent diffusion coefficient; Chemoembolization

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INTRODUCTION

Transcatheter arterial chemoembolization (TACE) is a kind of classic interventional therapy and it is commonly performed to treat the unresectable hepatocellular carcinoma and secondary liver cancers. The major goal of chemoembolization is to destroy the tumor. It is very important to evaluate obviously progression of hepatic tumors and differentiate accurately the areas and degrees of necrotic tumor from that of viable tumor before and after chemoembolization.

As reported, ultrasound (US), digital subtraction

angiography (DSA), computed tomography (CT) and magnetic resonance imaging (MRI) are usually used to diagnose and evaluate the progression of hepatic tumor but they have their respective defects. US is able to comprehend the size, blood provision and liquefied or cystic areas of tumor but it can not differentiate necrotic tumor from viable tumor[1,2]. DSA can infer necrotic tumor and viable tumor from the degree of tumor stain. CT can manifest the areas of iodized oil and necrotic or viable tumor but, because density of iodized oil is very high on CT, some areas of necrotic or viable tumor are easily covered before and after chemoembolization[3-5]. MRI is not affected by high density of iodized oil and it is more valuable than US and CT in evaluating the progression of the tumor and in differentiating necrotic from viable tumor. The signals of coagulative necrotic areas of tumor are low on T1- and T2-weighted imaging after chemoembolization and there are no enhanced in MR enhancement scanning[6-11].

Diffusion-weighted imaging is a kind of new functional imaging technology having been developed in recent years and it is the only one method which is able to reflect non-woundingly water molecular diffusion *in vivo*. It has been generally accepted that it is valuable in diagnosing qualitatively and quantitatively cerebral ischemia in hyper-inchoate period[12-14] and, at the same time, many studies of hepatic pathological changes on DWI have been reported[15-17]. ADC values of benign lesions, such as hepatic cysts and hemangiomas, were higher than those of malignant lesions, such as hepatocellular carcinomas and metastases on DWI, as reported by Ichikawa *et al*[15,16], Yamashita *et al*[17], Taouli *et al*[18], Sun *et al*[19]. Colagrande *et al*[20], indicating that the signals of tumor coagulative necrotic areas were lower in comparison with those of tumor viable areas. Kamel *et al*[21] confirmed, in their clinical investigation of 8 case of hepatocellular carcinomas, by image-pathology, that ADC would become high directly with the degree of tumor cellular necrosis increasing and that the signals of 6 tumors were higher than those of normal parenchyma on DWI. Geschwind *et al*[22] demonstrated that the signals of VX-2 tumor necrotic areas were low and that ADC in the area of tumor necrosis were significantly greater than those in the area of viable tumor after chemoembolization.

As indicated by findings of above preliminary experiments, DWI, especially ADC, has potential values in reflecting characteristics of liver pathological changes and of differentiation to benign tumor from malignant one.

There has been no dynamically and image-pathologically investigated report on the characteristics of hepatocellular carcinomas on DWI after chemoembolization. The purpose of our experiment is to investigate dynamic characteristics and pathological mechanism of signal with DWI in rabbit VX-2 tumor model after chemoembolization, which is the most valuable animal model of hepatocellular carcinoma for imaging investigations. Moreover the aim is to evaluate the contribution of DWI in differentiating of tumor viable cells to necrotic ones.

MATERIALS AND METHODS

Animals and establishment of VX-2 tumor model

Animal studies were carried out under the supervision of a veterinarian according to the guidelines on the Use of Laboratory Animals of the Ministry of Public Health of China. All animals were provided by the Laboratory Animal Center of the Second Xiangya Hospital and all protocols were approved by the Animal Use and Care Committee of the Second Xiangya Hospital.

Forty New Zealand rabbits were included in the study. Twenty-two were male rabbits and eighteen were female, weighed 1.7 to 2.5 kg, aged 5 to 6 mo. All of them were healthy. Forty-seven rabbit VX-2 tumor models were raised by implanting the tumor directly and intrahepatically after the abdominal cavity was opened. The VX-2 tumor strain of rabbit was provided by the Fourth Military Medical University.

Forty VX-2 tumor models were layered and randomly chosen from forty-seven VX-2 tumor models and were divided into four groups, including control group (non-interventional group, namely group A) and investigation group (at the 16th h after chemoembolization, at the 32nd h after chemoembolization, at the 48th h after chemoembolization, namely group B, C, and D, respectively). Otherwise, ten cases were randomly chosen from all data were carried out by DWI for all rabbits of group B, C and D at 6th h after chemoembolization and the data was put into group of the 6th h after chemoembolization, as one of investigation groups, namely E group.

Chemoembolization protocol

After DWI at the 21st d after implantation, trans-hepaticartery catheterization chemoembolization was directly and respectively carried out for all rabbits of B, C and D group in the Animal Operating Room of the Second Xiangya Hospital.

First, once rabbits were anesthetized by injecting 3% soluble pentobarbitone and the skin of abdomen was disinfected, then we exposed organs of the hepatic hilar region by incising the skin and vagina musculi recti abdominis. Arteria coeliaca, arteria hepatica communis, arteria hepatica propria, arteria gastroduodenalis, portal vein *etc* were recognized. Second, we drew off arteria gastroduodenalis, we occluded its distalpart and dropped a little attenuated aethocaine on it. Third, we punctured the arteria gastroduodenalis and put plastic transfixion pins or scalp acupunctures into it; the top of transfixion pins were put in arteria hepatica propria. Fourth, we fixed the microtubular or transfixion pins and then infused iodized oil (0.3 mL/kg) and pharmorubicin (2 mg/kg) into arteria hepatica. At last, abdominal membrane, musculature and cutaneous were sutured layer by layer after the liver and other organs in abdominal cavity stopped bleeding.

Magnetic resonance imaging protocol

After animals were anesthetized by injecting 3% soluble pentobarbitone into auriborder vein at a dose of 1 mL/kg or at different doses based on different animal status to make sure that the breathing of animals was slow and stable, DWI(axial) was carried respectively and periodically out a 1.5-Tesla Signa Twinspeed MR scanner (General Electron Medical Systems, USA) using a small diameter cylindrical brain radiofrequency coil before chemoembolization and at the 6th h, the 16th h, the 32nd

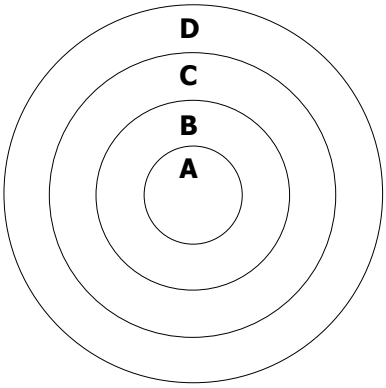

Figure 1 **A**: The area of VX-2 tumor center; **B**: The area of VX-2 tumor periphery; **C**: The area of VX-2 tumor outer layer; **D**: The normal liver parenchyma area around tumor when the values of ADC and signals were measured on DWI and samples were investigated pathologically.

h and the 48th h after chemoembolization. The scanning parameters of DWI included spin echo echoplanar imaging (SE-EPI) series, b-value 100 and 300 s/mm^2, repetition time (TR) 6000 ms, echo time (TE) 45 ms, 20 cm × 15 cm field of view (FOV), 8 number of excitations (NEX), 2 mm thickness layer, 0.5 mm Space, 128 × 128 matrix, *etc*.

ADC values and signal values in the area of VX-2 tumor periphery, in the VX-2 tumor center and in the normal liver parenchyma around tumor (Figure 1) were obtained by using Function Software in GE workstation. Three different regions of interest (ROIs) (50 mm^2 each area) were chosen in the area of normal liver parenchyma (area D in Figure 1) and we measured their ADC values and signal values. The average value of above was considered as the ADC value or the signal value of normal liver parenchyma around tumor. The thickness of area A and B in Figure 1 was respectively two fifth diameter of VX-2 tumor. By the same methods, the average value of three different ROIs ADC values or signal values in area B was considered as the ADC value or the signal value of VX-2 tumor periphery area; ADC value or signal value of area A was its ADC value or signal value of VX-2 tumor center area. All measurements were finished cooperatively by two senior attending physicians or associate professors.

Pathology protocol

All of the rabbits in each group were euthanized by injecting an overdose of 3% soluble pentobarbitone into auriborder vein when DWI was respectively carried out before chemoembolization, and after the 16 h, the 32 h and 48 from chemoembolization. We got layer by layer VX-2 samples under the condition of asepsis (Figure 1) and made them fixed in the formaldehyde solution for 24 h before being embedded in mineral wax. Each VX-2 tumor was divided into the outer layer area, the periphery area and the center area (Figure 1) so that samples included four parts: VX-2 tumor center area, VX-2 tumor periphery area, VX-2 tumor outer layer area and normal liver parenchyma around tumor.

All samples were investigated respectively under 100 × and 400 × microscope and the emphases were on investigating cellular edema in the VX-2 tumor and normal liver parenchyma around tumor.

Edema index was used to estimate the degree of cellular edema, which was the contrast of edema cell number to total cells under microscope. Manifestations of edema cells under microscope included cell body increasing, unclear cellular membrane and intracytoplasm vacuolar or ballooning degeneration; the latter was the most important manifestation of edema cell. Two campus visualises under 400 × microscope were obtained randomly in the zone of non-necrosis. The number of edema cells and total cell number were counted by two external doctors not from our study team with double blind method. Otherwise, we investigated specially the degree of tumor cellular necrosis and the abnormity of cell membrane. Statistical analysis based on apparent diffusion coefficient (ADC) value of ROIs and edema index, the distinction between different area groups, different time groups and different b-value groups was respectively estimated. The statistical significance was calculated by analysis of variance (ANOVA) or analysis of non-parameter by using SPSS software (version 12.0; SPSS, Tokyo, Japan)

RESULTS

Image manifestations of hepatic VX-2 tumor before and after chemoembolization

ADC values and signal values of VX-2 tumors were shown in Table 1 and Figures 2-5.

The distinction between ADC in the area of VX-2 tumor periphery, tumor center or normal parenchyma around tumor was respectively significant ($F = 14.366$, $P < 0.001$; $F = 4.674$, $P = 0.033$; $F = 23.054$, $P < 0.001$) with b-value 100 s/mm^2 and with b-value is 300 s/mm^2 was respectively significant ($F = 14.366$, $P < 0.001$; $F = 4.674$, $P = 0.033$; $F = 23.054$, $P < 0.001$). Signals in the area of VX-2 tumor periphery, tumor center and normal parenchyma around tumor b-value was 100 s/mm^2 were higher than with b-value was 300 s/mm^2 ($F = 112.874$, $P < 0.001$; $F = 83.455$, $P < 0.001$; $F = 135.455$, $P < 0.001$).

When b-value was 100 s/mm^2, the distinction of ADC in the area of VX-2 tumor periphery, tumor center and normal parenchyma around tumor among group A, B, C, D and E was respectively significant($F = 7.325$, $P < 0.001$; $F = 2.496$, $P = 0.048$; $F = 6.856$, $P < 0.001$). The distinction of signal in the area of VX-2 tumor periphery among group A, B, C, D and E was significant ($F = 3.005$, $P < 0.05$) but that in the area of VX-2 tumor center and normal parenchyma around tumor was not significant ($F = 1.399$, $P > 0.05$; $F = 2.146$, $P > 0.05$).

Manifestations of VX-2 tumor pathology

Observed by the naked eye, most surfaces of normal hepatic parenchyma around VX-2 tumor were paler in investigation group than those in control group and there was embolization of unequal areas but well-circumscribed. The tumors were hard and there was clear demarcation. The cavities of unequal size were found in the area of VX-2 tumors because kermesinus liquid had run off after the tumors werfae cut open.

Under microscope, some edema cells containing ballooning degeneration were observed in the area of normal parenchyma around VX-2 tumor. Inequality

Table 1 ADC values of tumor and normal parenchyma after Chemoembolization

Group	VX-2 tumor periphery areas		VX-2 tumor center areas		Hepatic normal parenchyma	
	b = 100	b = 300	b = 100	b = 300	b = 100	b = 300
Control	1.71 ± 0.27	1.48 ± 0.23	1.77 ± 0.36	1.55 ± 0.30	2.71 ± 0.42	2.30 ± 0.40
6 h	1.56 ± 0.40	1.36 ± 0.18	1.97 ± 0.49	1.79 ± 0.37	2.44 ± 0.53	1.87 ± 0.31
16 h	1.24 ± 0.22	1.12 ± 0.20	1.56 ± 0.40	1.69 ± 0.35	2.10 ± 0.54	1.65 ± 0.37
32 h	1.48 ± 0.37	1.23 ± 0.16	1.99 ± 0.32	1.66 ± 0.31	2.10 ± 0.49	1.97 ± 0.29
48 h	1.57 ± 0.23	1.40 ± 0.18	2.04 ± 0.54	1.82 ± 0.27	2.43 ± 0.33	2.06 ± 0.23
Total	1.51 ± 0.33	1.32 ± 0.23	1.87 ± 0.45	1.70 ± 0.32	2.36 ± 0.51	1.97 ± 0.38

Data are expressed as mean ± SD × 10^{-3} mm^2/s; ADC: Apparent diffusion coefficient.

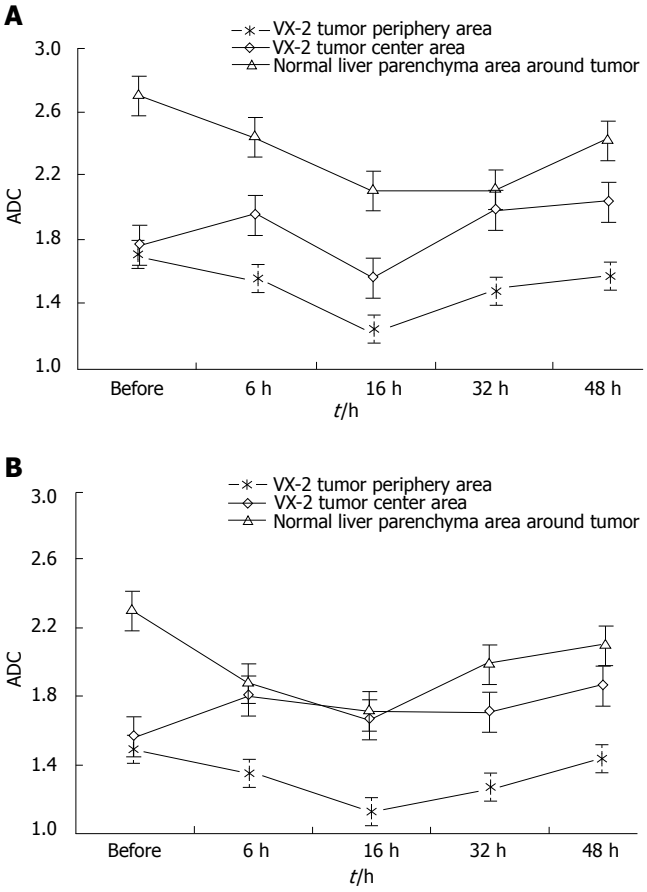

Figure 2 ADC values of different areas on DWI after chemoembolization. **A**: B-value was 100 s/mm^2; **B**: B-value was 300 s/mm^2.

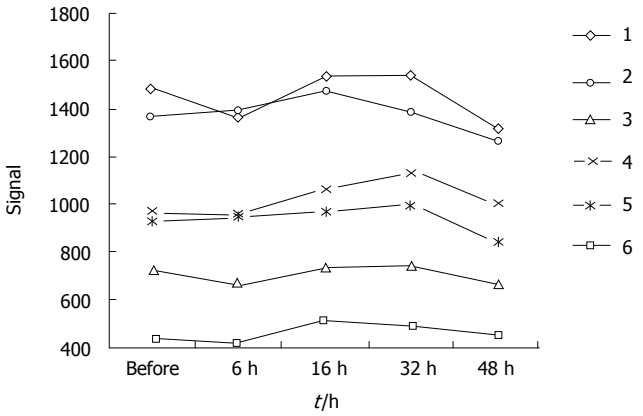

Figure 3 Signal values on DWI when area and b-value was different after chemoembolization. Areas: 1-signal of VX-2 tumor periphery area when b-value was 100 s/mm^2; 2-signal of VX-2 tumor center area when b-value was 100 s/mm^2; 3-signal of the normal liver parenchyma area around tumor when b-value was 100 s/mm^2; 4-signal of VX-2 tumor periphery area when b-value was 300 s/mm^2; 5-signal of VX-2 tumor center area when b-value was 300 s/mm^2; 6-signal of the normal liver parenchyma area around tumor when b-value was 300 s/mm^2.

of size, round or ellipse or strip tumor nests could be observed in different areas of VX-2 tumor. The degree of cell edema and necrosis were more obvious in group B,C and D (after chemoembolization) than group A (before chemoembolization), the necrotic areas were more in the area of VX-2 tumor center than those in the area of tumor periphery or tumor outer layer, and most areas of tumor center were necrotic in some VX-2 tumors.

Edema cells showed their volume increased obviously, kytoplasm dyeing thinly and obvious ballooning degeneration (Figure 6 and 7). Dynamical information of cellular edema and necrosis were counted in Tables 2 and 3.

DISCUSSION

The signal and ADC characteristics of many hepatic pathological changes on DWI have been reported in recent years[23-26]. DWI has significant and potential clinical application values in detecting, diagnosing and differentiating tumors earlier. From what has been determined when DWI was investigated by Yuan *et al* in rabbit liver VX-2 tumor model, VX-2 tumor is a solid tumor and its body mainly consists of tumor nests and other cells so that its water molecule diffusion motion is obviously restricted, the signal of them is significantly high and ADC is significantly low.

The signals in VX-2 tumors were higher than those in the area of normal parenchyma around tumor while ADC values were lower than those in the area of normal parenchyma after chemoembolization, and the tendency of the signal and ADC in VX-2 tumors and in the area of normal parenchyma was basically the same. The signals in VX-2 tumors were uneven, the signals in the area of tumor center were lower than that in the area of tumor periphery, while those in the 48 h after chemoembolization were lower than that before chemoembolization. The areas of low signal and high signal were observed in VX-2 tumor and ADC of them was higher than that in the equal signal area of VX-2 tumor or in the area of normal parenchyma. Accordingly with pathology, the areas of low signal in VX-2 tumor were coagulation necrosis, those of high signal were liquid or cystic tissue,

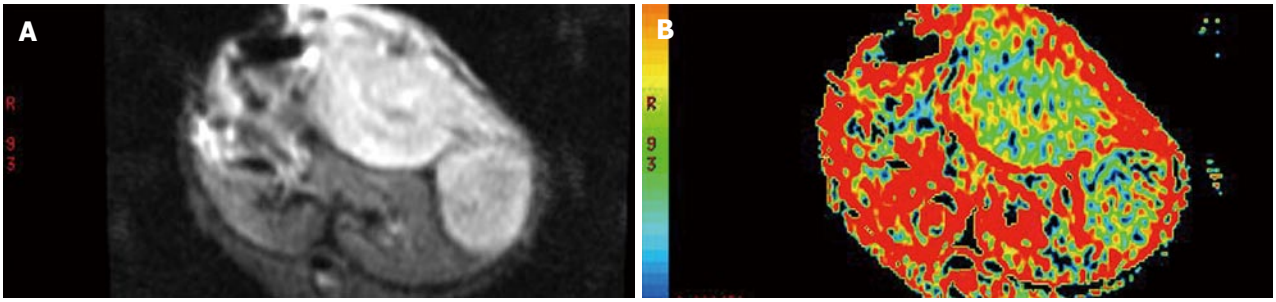

Figure 4 Image manifestations of hepatic VX-2 tumor on DWI and ADC map when b-value was 100 s/mm^2 at 6 h after chemoembolization **A**: High signal and distinct margin of VX-2 tumor on DWI; **B**: Low signal of it on the ADC map.

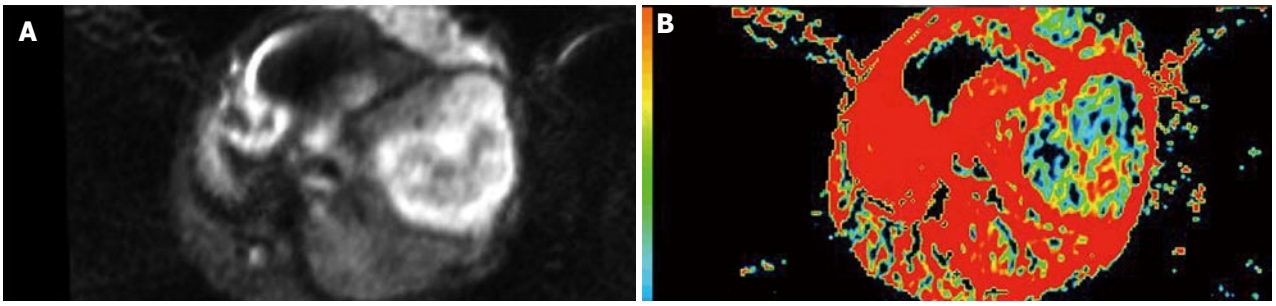

Figure 5 Image manifestations of hepatic VX-2 tumor on DWI and ADC map when b-value was 100 s/mm^2 at 48 h after chemoembolization **A**: High and uneven signal and distinct margin of VX-2 tumor on DWI; **B**: Low and uneven signal of it on the ADC map.

and other areas except low signal and high signal areas in the lump were viable tumor cells. Moreover, necrotic areas in VX-2 tumors after chemoembolization were more than those before chemoembolization. When coagulation necrosises/necroses takes place in the tumor because of insufficient blood provision, cellular membrane will break and the limitation of water molecular motion in the tumor decreases greatly so that the signals is reduced while ADC values are upgraded. After coagulation necroses have been liquefied or become cystic, cell lysis and leakier cell membranes can no longer compartmentalize water molecules and allow free diffusion to take place so that ADC values increase greatly. Nyway the signals of above mentioned liquefied or cystic areas are higher than those of coagulative necrosis areas, even viable tumor areas or normalparenchyma. It can be explained by the presence of greater amounts of extracellular water molecules within the necrotic region which is a kind of long T2-value contribution constitution and the b-value is 100 or 300 s/mm^2, a small b-value, in diffusion-weighted imaging scanning so that the signals of above are affected significantly by "shine-through".

At 6 h after chemoembolization, the signals in the area of tumor centers decreased slightly while ADC increased slightly, the signals in the area of tumor periphery and normal parenchyma increased significantly while ADC decreased at different degrees. However, pathology demonstrated that the degree of cellular necrosis in the area of normal parenchyma before chemoembolization was the same of that after chemoembolization while in the area of tumor periphery areas before chemoembolization was much more than after chemoembolization.

Accordingly with what discussed above, the signal increasing and ADC decreasing cannot completely be explained by tumor cellular necrosis after chemoembolization. The dynamic mechanisms of water molecular diffusion decreasing and the signal increasing while ADC values decreasing after cerebral infarction have been investigated by many investigators in recent years, and it is not completely comprehended yet, but most of them demonstrated it was connected with cytotoxic edema, microcirculation disturbance, temperature or abnormality of cellular membrane permeability, *etc*[27-31]. A series of clinical and animal experimental investigations by Xie *et al*[28], Han *et al*[29] and Marks *et al*[30] have demonstrated that the cytotoxic edema after embolization had significant influences on the change of water molecules diffusion. Because of dysfunctional Na-K pump due to early hypoxia after embolization, the density of intra-cellular electrolyte increases and then also water molecules of intra-cells increase significantly, while extra-cell water molecules decrease significantly, so that ADC starts decreasing while the signal starts increasing. When intracellular edema reaches to the biggest degree, ADC of constitution will decrease to the lowest and it will maintain at a low degree if intracellular edema maintains or if there are constitution edema originating from the blood vessel. ADC of constitution will start increasing after cellular membrane has been broken and cells have dissolved; it will reach the biggest when constitution has been liquefied and become cystic.

After chemoembolization, ADC in the area of VX-2 tumor periphery and normal parenchyma decreased quickly wuth ther bottom at biggest in the 16th h and

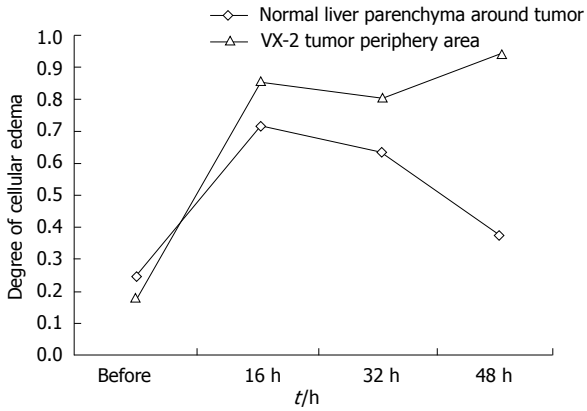

Figure 6 Cellular edema of different areas after chemoembolization.

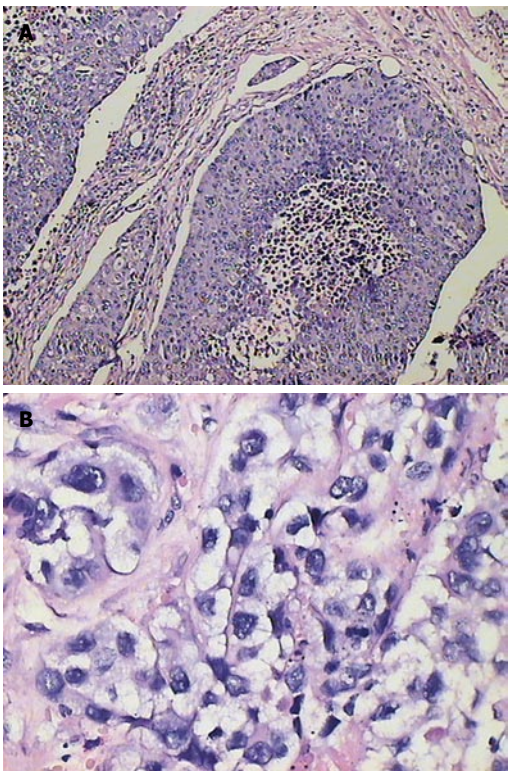

Figure 7 **A**: Cell nest of VX-2 tumor and wide zone of necrosis in the tumor(x 100) and **B**: cellular edema in the VX-2 tumor (x 400).

then increased gradually but they were significantly lower in the 48th h after chemoembolization than those before chemoembolization. Pathology demonstrated the degree of cellular edema in the area of VX-2 tumor and normal parenchyma were significant higher after chemoembolization than before chemoembolization and it reaching a peak at 16th h after chemoembolization and then decreased gradually. The signal and ADC changes in the area of VX-2 tumor and normal parenchyma were significantly relative to cellular edema. The function of Na-K pump decreases or misses because of ischemia and hypoxia from chemoembolization and toxic action from chemotherapeutic drug; the degree of intra-cell edema also increases significantly after chemoembolization so that ADC starts decreasing. With intracellular edema lessening

Table 2 Dynamic information of cellular edema after chemoembolization (%) (mean ± SD)

	Control	16 h	32 h	48 h
Normal parenchyma	24.90 ± 11.69	72.12 ± 34.48	63.89 ± 29.87	38.00 ± 24.57
Tumor periphery areas	18.16 ± 10.35	86.06 ± 16.01	81.18 ± 20.03	96.66 ± 4.76

Table 3 Manifestations of VX-2 tumor pathology after chemoembolization

	Cellular membrane		Cellular necrosis			
	0	1	0	1	2	3
Normal parenchyma (Control)	8	2	7	3		
Tumor periphery areas (Control)	10				9	1
Normal parenchyma (16 h)	7	3	7	1	2	
Tumor periphery areas (16 h)	10			2	4	4
Normal parenchyma (32 h)	7	3	7	1	2	
Tumor periphery areas (32 h)	10				3	7
Normal parenchyma (48 h)	6	4	8	2		
Tumor periphery areas (48 h)	10				4	6

Cellular membrane: 0-clear and complete; 1-unclear and non-complete. Cellular necrosis: 0-cellular structure existing and cellular membrane complete; 1-many cells dissolving and cellular membrane disappearing; 2-minute cellular coagulation necrosis; 3-extensive cellular coagulation necrosis.

or cell breaking, ADC values will increase gradually. The dynamic changes of ADC can reflect the degree of tumor cellular edema and cellular necrosis.

However, after chemoembolization, ADC in the area of VX-2 tumor periphery and normal parenchyma increased gradually but the signals decreased gradually from the 16th h to the 48th h after chemoembolization; the signals were lower in the 48th h than those before chemoembolization. Pathology demonstrated that the degree of cellular edema in the area of normal parenchyma reached a peak in the 16th h and then decreased gradually but that in the area of tumor periphery reached a peak in the 16th h and then increased continually after chemoembolization. The degree of cellular edema in the area of tumor periphery was significantly lower than that in the area of normal parenchyma. Double blood-provision from hepatic artery and portal system plus our protocols of transcatheter hepaticarterial chemoembolization can explain it. Selective catheterization was not carried out in our experiments so that ischemia and hypoxia took place in the VX-2 tumor and normal parenchyma at the same time and ADC decreased because of intracellular edema. The degree of cellular edema in VX-2 tumor was higher than that in the area of normal parenchyma after chemoembolization because 95%-99% blood provision of hepatocellular carcinoma comes from hepatic artery while 70%-75% blood provision of normal parenchyma comes from portal system and others comes from hepatic artery. Blood provision from portal system would recover step by step after chemoembolization so that the degree of cellular edema in the area of normal parenchyma decreased significantly but that in VX-2

tumor increased continually from the 16 h to the 48 h after chemoembolization. Otherwise, as reported by Yang *et al*[31], blood provision decreasing could lead to ADC decreasing and ADC reflected blood provision to a certain degree when b-value was small in diffusion-weighted imaging scanning. Since b-value was small in our scanning, ADC could be affected by blood provision after chemoembolization.

Necrotic tumor manifests low signal and high ADC value, while viable tumor manifests high signal and low ADC value after chemoembolization. DWI has potential ability in detecting and differentiating viable tumor from necrotic tumor and, besides water molecular diffusion, intracellular edema, microcirculation disturbance and tumor necrosis from chemoembolization are significantly relative to ADC changing dynamically.

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COMMENTS

Background

Transcatheter arterial chemoembolization (TACE) is a kind of classic interventional therapy and it is commonly performed to treat the unresectable hepatocellular carcinoma and most secondary liver cancers. The major goal of chemoembolization is to destroy the tumor. Ultrasound (US), digital subtraction angiography (DSA), computed tomography (CT) and magnetic resonance imaging (MRI) are usually used to diagnose and to evaluate the progression of hepatic tumor but they have their respective defects. Diffusion-weighted imaging is a kind of new functional imaging technology having been developed in recent years and it is the only one method which is able to reflect non-woundingly water molecular diffusion *in vivo*. Geschwind *et al* demonstrated that the signals of VX-2 tumor necrotic areas were low and ADC of them were significantly greater in the area of tumor necrosis than those in the area of viable tumor after chemoembolization. Therefore, we believe that DWI, especially ADC, has potential values in reflecting characteristics of liver pathological changes and differentiating benign tumor from malignant one.

Research frontiers

Many studies of hepatic pathological changes on DWI have been reported. ADC values of benign lesions, such as hepatic cysts and hemangiomas, were higher than those of malignant lesions, such as hepatocellular carcinomas and metastases on DWI and many studies also indicated that the signals of tumor coagulative necrotic areas were lower in comparison with that of tumor viable areas. There has been no dynamically and image-pathologically investigated report on the characteristics of hepatocellular carcinomas on DWI after chemoem-bolization.

Innovations and breakthroughs

Our study clearly showed that necrotic tumor manifested low signal and high ADC value while viable tumor manifested high signal and low ADC value after chemoembolization. DWI would have potential ability in detecting and differentiating viable tumor from necrotic tumor and, besides water molecular diffusion, intracellular edema, microcirculation disturbance and tumor necrosis from chemoembolization were significantly relative to ADC changing dynamically.

Applications

Physicians can apply this knowledge to evaluate obviously progression of hepatic tumors and differentiate accurately the areas and degrees of necrotic tumor from that of viable tumor before and after chemoembolization.

Peer review

This is an interesting, well designed, and written study on a problem of real clinical significance.

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BASIC RESEARCH

Concurrent repletion of iron and zinc reduces intestinal oxidative damage in iron- and zinc-deficient rats

Sreedhar Bodiga, Madhavan Nair Krishnapillai

Sreedhar Bodiga, Madhavan Nair Krishnapillai, Department of Biophysics, National Institute of Nutrition, Hyderabad 500007, Andhra Pradesh, India
Supported by the Indian Council of Medical Research
Correspondence to: Dr. Madhavan Nair Krishnapillai, Department of Biophysics, National Institute of Nutrition, Indian Council of Medical Research, Jamai-Osmania PO, Hyderabad 500007, Andhra Pradesh, India. nairthayil@hotmail.com
Telephone: +91-40-27008921 Fax: +91-40-27019074
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Abstract

AIM: To understand the interactions between iron and zinc during absorption in iron- and zinc-deficient rats, and their consequences on intestinal oxidant-antioxidant balance.

METHODS: Twenty-four weanling Wistar-Kyoto rats fed an iron- and zinc-deficient diet (< 6.5 mg Fe and 4.0 mg Zn/kg diet) for 4 wk were randomly divided into three groups (n = 8, each) and orally gavaged with 4 mg iron, 3.3 mg zinc, or 4 mg iron + 3.3 mg zinc for 2 wk. On the last day of repletion, 3 h before the animals were sacrificed, they received either 37 mBq of ^{55}Fe or ^{65}Zn, to study their localization in the intestine, using microautoradiography. Hemoglobin, iron and zinc content in plasma and liver were measured as indicators of iron and zinc status. Duodenal sections were used for immunochemical staining of ferritin and metallothionein. Duodenal homogenates (mitochondrial and cytosolic fractions), were used to assess aconitase activity, oxidative stress, functional integrity and the response of antioxidant enzymes.

RESULTS: Concurrent repletion of iron- and zinc-deficient rats showed reduced localization of these minerals compared to rats that were teated with iron or zinc alone; these data provide evidence for antagonistic interactions. This resulted in reduced formation of lipid and protein oxidation products and better functional integrity of the intestinal mucosa. Further, combined repletion lowered iron-associated aconitase activity and ferritin expression, but significantly elevated metallothionein and glutathione levels in the intestinal mucosa. The mechanism of interactions during combined supplementation and its subsequent effects appeared to be due to modulation of cytosolic aconitase, which in turn influenced the labile iron pool and metallothionein levels, and hence reduced intestinal oxidative damage.

CONCLUSION: Concurrent administration of iron and zinc corrects iron and zinc deficiency, and also reduces the intestinal oxidative damage associated with iron supplementation.

Key words: Iron; Zinc; Absorption; Intestine; Aconitase; Ferritin; Metallothionein; Oxidative damage

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INTRODUCTION

Iron-deficiency anemia is frequently the result of low intake of dietary iron, and high intake of phytate and tannins, which inhibit iron absorption. An iron-deficient population can generally have zinc deficiency, due to similar influences of various dietary factors on iron and zinc absorption[1]. There is strong evidence in humans that a blockage of zinc absorption is a consequence of daily intake of high amounts of iron[2]. Studies in pregnant women also provide evidence that the absorption of supplemental iron is lower from multimineral antenatal supplements, particularly in the presence of high calcium and magnesium than when it is administered alone[3,4]. It has therefore become evident during the past decade that deficiencies of iron and zinc co-exist, and those vulnerable groups may benefit from iron as well as zinc supplementation, rather than individual supplementation with these minerals. However, iron and zinc are known to interact, either at the site of absorption or post-absorptively, because of competition for similar transport pathways[5-8]. Moreover, these two minerals have opposing effects on oxidant-antioxidant balance in either iron- or zinc-deficient intestines[8,9]. As mineral supplementation programs are designed for populations at risk of both iron and zinc deficiencies, understanding of quantitative and qualitative aspects of the potential of the two metals is critical. Iron and zinc have been found to interact antagonistically at the site of absorption, and this interaction helps in reducing the iron-mediated generation of hydroxyl radicals and

subsequent intestinal oxidative damage during repletion in a single mineral deficiency[8,9]. Other cascading effects of such interactions are the reduction of iron and zinc uptake, aconitase activity, and ferritin expression in the intestinal mucosa. Besides, zinc induction of metallothionein in the intestinal mucosa helps to restore its functional integrity[8]. However, absorption of these two minerals is regulated at the intestinal level[10,11], and hence the nature and extent of interactions may differ when the deficiencies co-exist, because of molecular adaptations that modulate the absorption of these minerals. Therefore, in the present study, we depleted iron and zinc in weanling rats and studied the nature of the interactions between two metals, and their consequences on intestinal oxidant-antioxidant balance; finally we postulated a probable mechanism.

MATERIALS AND METHODS

Animals and diets

This study employed a typical depletion-repletion design for understanding the potential interactions of iron and zinc during supplementation in iron- and zinc-deficient rats. The Institutional Animal Ethical and Bio-safety Committee of the National Institute of Nutrition approved the study.

Twenty-four weanling female Wistar-Kyoto rats, (National Center for Laboratory Animal Science at the National Institute of Nutrition, Hyderabad, India) weighing 35-45 g, were fed an egg-albumin-based, semi-synthetic, purified iron- and zinc-deficient diet (< 6.5 mg Fe and 4.0 mg Zn/kg diet) for 4 wk (Table 1). Rats were housed individually in polypropylene cages with stainless steel wire floors (45 cm × 16 cm, 7.5 mm mesh, 1 mm wire diameter) to prevent coprophagy, in a room maintained at 23℃ and 60% humidity, with a 12-h light: dark cycle. Deionized distilled water, in plastic bottles with stainless steel sippers, was freely available to all rats. Body weight was recorded weekly and blood was collected by orbital sinus puncture for determination of iron and zinc status at the end of depletion and repletion.

Oral repletion of iron and/or zinc

At the end of depletion phase, rats were randomly divided into three groups and assigned to either Fe, Zn and Fe + Zn groups for repletion (n = 8, each). In order to achieve complete repletion of both the minerals, while minimizing the intestinal oxidative damage, we used a dose of 4 mg iron and 3.3 mg zinc for repletion. Force feeding was performed daily during the repletion period, either with a dose of 4.0 mg iron (Fe group), 3.3 mg zinc (Zn group) or a combination of 4.0 mg iron and 3.3 mg zinc (Fe + Zn group) in 1.0 mL 0.01 mol/L HCl, via an orogastric plastic tube, for 2 wk[12]. During this period, the rats were fed an iron- and zinc-deficient diet *ad libitum*. The doses were prepared by dissolving $FeSO_4$ and $ZnSO_4$ salts, either individually or in combination, in dilute HCl[8]. Administration of 0.01 mol/L HCl alone to the rats had no significant effect on the parameters studied[12]. Therefore, vehicle controls were not included in the present study. All the rats were fasted for 16 h before administering the last dose for uptake studies and for studying various other parameters.

Table 1 Composition of the iron and zinc-deficient diet

Ingredient	Concentration g/kg diet
Egg albumin powder	220
Cornstarch	293
Sucrose	300
Cellulose	40
Peanut oil	70
Mineral mixture[1]	35
Vitamin mixture[1]	10
DL-methionine	4

[1]Mineral and vitamin mixture were formulated according to AIN (1993), but mineral mixture was devoid of iron and zinc salts (4.0 mg Zn and 6.5 mg Fe/kg diet) during depletion and repletion.

Uptake of iron and zinc; immunolocalization of ferritin and metallothionein in the duodenum

Three rats, each from the Fe and Zn repleted groups, along with six rats from the Fe + Zn repleted group (three each for ^{55}Fe and ^{65}Zn) were randomly selected. Fe group rats received 4.0 mg Fe with 37 mBq of ^{55}Fe (specific activity, 46.9 mCi/g), Zn group rats received 3.3 mg Zn with 37 mBq of ^{65}Zn (specific activity, 1.14 Ci/g), and the Fe + Zn group rats received 4.0 mg Fe and 3.3 mg Zn with either 37 mBq of ^{55}Fe (n = 3) or 37 mBq of ^{65}Zn (n = 3) (BRIT, Mumbai, India). The rats were killed after collecting blood. Proximal 10-cm portion of the duodenum was excised for mucosa collection. Samples of liver were collected from the rats for iron and zinc determination by atomic absorption spectrophotometry.

The tissues were placed immediately on ice, trimmed of excess fat and mesentery, and weighed. The intestinal segments were flushed with ice-cold saline. Of the 10-cm portion, the first 2.0 cm was used for studying iron and zinc uptake, and localization of their responsive proteins, while the remaining portion was saved and processed for obtaining mucosal homogenates. The 2.0-cm portion was placed in 10% neutral-buffered formalin for 12 h, washed in PBS for 24 h, using a "Swiss roll" technique[13] to evaluate the entire longitudinal section on one slide and embedded in paraffin at 58℃. Multiple serial sections of 4-μm thickness were obtained from these blocks. These sections were dewaxed, defatted, mounted on chrome alum-gelatin coated slides, and dip-coated with Amersham LM1 photographic emulsion (Amersham, UK). These were then exposed for 30 d in a light-proof desiccator at 4℃, before developing and fixing in Kodak D-19 developer and Kodak Rapid fix (Kodak, Rochester NY), respectively. Sections were stained with Mayer's hematoxylin and eosin, and then examined using a Nikon Microfot-FX microscope (Nikon, Japan) at 100 × magnification, and photographed. In separate serial sections, localization of ferritin and metallothionein was probed using immunohistochemistry. Briefly, dewaxed and defatted sections were incubated overnight with a 1:500 dilution of rabbit anti-rat liver ferritin[12], or 1:50 dilution

of metallothionein antibody (Clone E9; Dako Corp, Carpinteria, CA), or with IgG from non-immune rabbit serum. The specific binding was detected by biotinylated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Sigma, St. Louis, MO) using hydrogen peroxide and diaminobenzidine. The endogenous peroxidase was quenched with 0.3% hydrogen peroxide in 100% methanol for 15 min. The sections were counterstained with hematoxylin and eosin and observed by light microscopy at 250 × magnification, and photographed.

Preparation of intestinal mucosa

We used the remaining 7-cm intestinal segment from the radioisotope-treated animals and the whole segment from non radioisotope-treated animals, for preparation of intestinal homogenates. All the animals were sufficiently repleted for 2 wk. However, radioisotope-treated animals received an additional dose of iron and/or zinc at the end of repletion. This single additional dose is unlikely to contribute much to the nutrient status compared to animals that have not received radioisotope.

The duodenal segments collected from each rat in ice-cold 1.15% KCl were scraped and a 10% homogenate of the mucosal suspension was prepared in 1.15% KCl containing 0.5 mmol/L butylated hydroxy tolune (to prevent *ex vivo* peroxidation) as described previously[12]. The homogenate was subjected to differential centrifugation (Sorvall RC-5B, Du Pont Instruments, CT) at 800 *g* for 30 min, 12 000 *g* for 30 min, and 100 000 *g* for 1 h at 4℃, and the respective supernatants were collected and stored at -20℃ until further analysis. The protein content in these supernatants was determined using a modified Lowry method, with BSA as the standard.

Iron and zinc content in diet, plasma and liver

Iron and zinc concentrations in the diet, liver, mineral solutions and plasma were determined by atomic absorption spectrophotometry using a SpectrAA-400 atomic absorption spectrometer (Varian, Sunnyvale, Melbourne, Australia). The mineral deficient diet fed to the rats was digested with concentrated nitric acid and 70% perchloric acid by method (Ⅱ) A of the Analytical Methods Committee (1960)[14]. One g of liver tissue was ashed in a muffle furnace at 600℃ for 6 h and was used to prepare the mineral solution.

Markers of functional integrity

Activity of alkaline phosphatase and lys-ala-dipeptidyl aminopeptidase was measured in the 12 000 *g* supernatant to assess the functional integrity of the intestinal mucosa, because of the differential localization of these two enzymes within the mucosal cells: the maximum activity of the former towards the upper half of the villus, and that of the latter towards the lower half[8,12]. Alkaline phosphatase activity was determined using β-glycerophosphate and lys-ala-dipeptidyl aminopeptidase activity was measured using lys-ala-7-amido-4-methyl coumarin.

Markers of oxidative stress

The amount of thiobarbituric acid-reactive substance (TBARS) in the 12 000 *g* supernatant was measured using malondialdehyde as a standard, and was quantified, using a reverse-phase silica-based C18 column, according to Templar *et al*[15]. Elution was done for 10 min at a flow rate of 1.2 mL/min with 65% 50 mmol/L KH_2PO_4-KOH, pH 7.0, and 35% methanol buffer, while monitoring at 532 nm. Protein carbonyls were estimated by incubation of 1 mL of the 100 000 *g* supernatant with 10 mmol/L dinitrophenyl hydrazine (DNPH) for 1 h in the dark, followed by protein precipitation with trichloroacetic acid according to the method of Reznick & Packer[16]. The final DNPH and lipid-free precipitate was dissolved in 6 mol/L guanidine hydrochloride and the absorbance was read at 375 nm. The amount of protein in the final pellet was quantified at 280 nm using BSA in guanidine hydrochloride as a standard.

Antioxidant status

Activity of total superoxide dismutase (SOD), glutathione peroxidase (100 000 *g* supernatant), Mn-SOD and catalase (12 000 *g* supernatant) was determined as previously described[17-19]. Glutathione (reduced GSH and oxidized GSSH) levels were determined using HPLC equipped with a fluorimetric detector (excitation at 350 nm and emission at 420 nm), according to Pastore *et al*[20]. Briefly, thiols were extracted using sulfosalicylic acid and injected onto a 150 mm × 4.6 mm Hypersil ODS column, equilibrated with 30 mmol/L ammonium nitrate and 40 mmol/L ammonium formate buffer, pH 3.6. For GSH determinations, $NaBH_4$ was substituted with 2 mmol/L EDTA-DTT. The thiols were eluted from the column with a 6-min gradient of acetonitrile (0-4 min, 0%-30% acetonitrile; 4-5 min, 30%-100% acetonitrile; 5-6 min, 100% acetonitrile) at a flow rate of 1.5 mL/min.

Activity of duodenal mucosal aconitase

The activity of aconitase in cytosolic (100 000 *g* supernatant) and mitochondrial (12 000 *g* pellet) was measured by a coupled isocitrate dehydrogenase reaction, monitored at 37℃ (Cary 100 Bio UV-Vis spectrophotometer, Varian Inc., CA) at 340 nm for 1 h [21,22].

Statistical analysis

Statistical analysis was performed using SPSS/PC+, version 5.0 (SPSS, Chicago, IL, USA). Comparison among the three groups was tested by one-way ANOVA with post-hoc multiple comparison tests. For measurements of body weight and iron and zinc status, comparisons were made with littermates maintained in the animal colony. The values were considered significantly different at $P < 0.05$.

RESULTS

Body weight changes

Rats fed an iron- and zinc-deficient diet for 4 wk weighed 57% less than their littermates in the animal colony (62.0 ± 8.0 g and 108.2 ± 7.5 g, respectively). However, the final bodyweight of Fe-, Zn- and Fe + Zn-repleted rats was similar, but weighed 50% of their littermates (95.6 ± 10.2 g and 190.0 ± 14.8 g).

Iron and zinc status

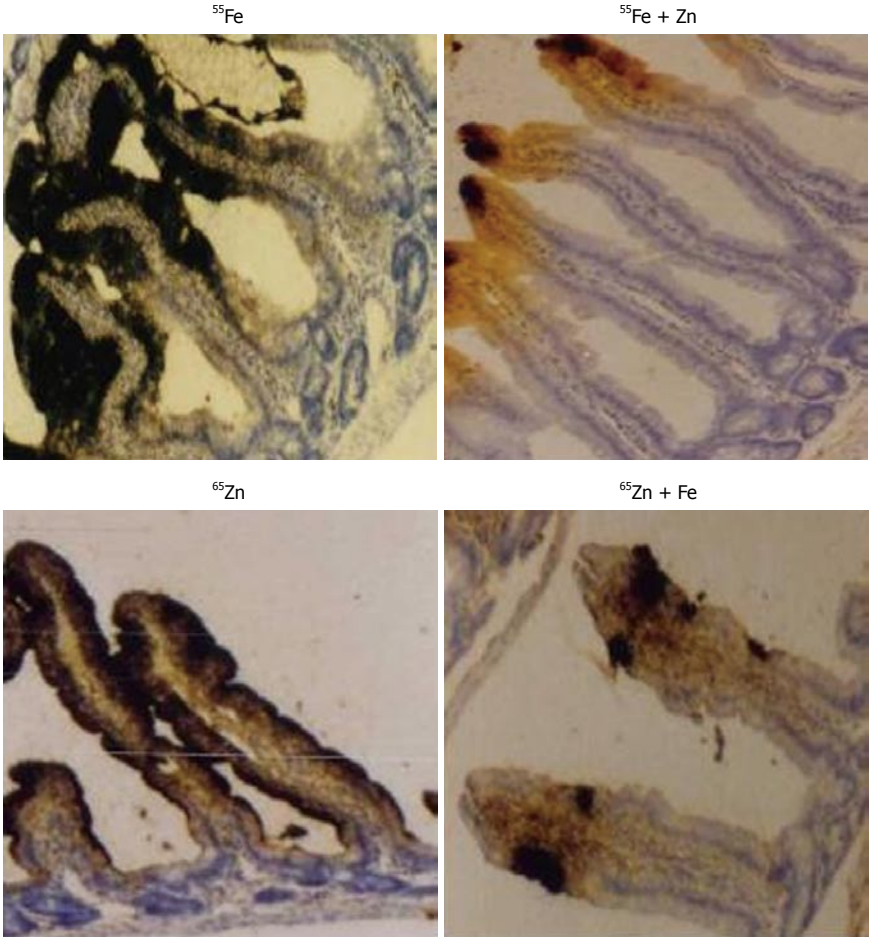

Figure 1 Zn reduces uptake of ^{55}Fe and Fe reduces uptake of ^{65}Zn during combined administration: A representative microautoradiogram of the duodenal mucosa of ^{55}Fe in Fe (top left), Fe + Zn (top right), ^{65}Zn in Zn (bottom left). and Fe + Zn (bottom right) groups. Iron and zinc deficient rats were orally administered 37mBq of ^{55}Fe and 4.0 mg Fe and/or 37mBq of ^{65}Zn and 3.3 mg Zn. Presence of black spots in the intestinal mucosa indicates the presence of the radioactivity (× 100).

Table 2 Iron and zinc status after concurrent depletion and repletion

Group	Hemoglobin (g/L)	Iron		Zinc	
		Plasma (μmol/L)	Liver (μg/g)	Plasma (μmol/L)	Liver (μg/g)
-Fe - Zn	82.0^{a} ± 3.2	65.0^{a} ± 4.6	-	12.3^{a} ± 2.0	-
Fe	126.9^{b} ± 3.0	94.8^{b} ± 7.2	192.8^{a} ± 12.5	13.0^{a} ± 1.8	22.8^{a} ± 4.5
Zn	80.0^{a} ± 2.8	64.0^{a} ± 5.5	43.7^{b} ± 6.6	29.6^{b} ± 3.0	48.6^{b} ± 4.6
Fe + Zn	106.8^{c} ± 3.0	82.4^{c} ± 3.8	157.5^{c} ± 7.7	20.5^{c} ± 3.2	37.1^{c} ± 4.2

Iron and zinc deficient (-Fe-Zn) rats were administered 4.0 mg Fe and/or 3.3 mg Zn/day for 2 wk; Fe: Iron administered group; Zn: Zinc administered group; Fe + Zn: Iron and zinc administered group; Number of animals was 8 in each group. Values are mean ± SD; means with different superscripts are significantly different with $P < 0.05$.

Iron and zinc depletion were confirmed by lowered hemoglobin and plasma zinc concentrations compared to their littermates in the animal colony, which were fed an iron- and zinc-adequate diet (35.0 mg Fe and 30.0 mg Zn/kg diet). Concurrent depletion of iron and zinc resulted in reduction of hemoglobin to 58%, plasma iron to 86% and plasma zinc to 38% compared to littermates. A significant lowering in all the above measured variables indicated the co-existence of iron and zinc deficiency in the experimental rats (Table 2). On repletion, hemoglobin and plasma and liver iron concentrations were significantly higher in the Fe and Fe + Zn groups compared to the Zn group (Table 2). Among the iron-repleted groups, iron status was significantly higher in the Fe than in the Fe + Zn group. Similarly, plasma and liver zinc concentrations were significantly higher in the Zn and Fe + Zn groups than in the Fe group. However, the Fe + Zn group showed significantly lower zinc status compared to the Zn group (Table 2).

Uptake of ^{55}Fe and ^{65}Zn; immunolocalization of ferritin and metallothionein in intestinal mucosa

To understand the effect of interactions following concurrent repletion of iron and zinc to depleted rats, we looked for their presence in the site of absorption. Uptake of ^{55}Fe and ^{65}Zn at the end of repletion in the intestinal mucosa was reduced in Fe + Zn-supplemented rats compared with Fe- or Zn-supplemented rats, respectively (Figure 1). Intestinal ferritin concentration was higher in the Fe than in the Fe + Zn group (Figure 2, upper panel). Identically, metallothionein concentration in the intestinal mucosa was high and appeared similar in the Zn and Fe + Zn groups, but relatively weak in the Fe group (Figure 2, Lower panel).

Intestinal oxidative stress and its functional integrity

Interactions between iron and zinc and their functional relevance to intestinal oxidative stress were assessed by as TBARS and protein carbonyls. TBARS and protein carbonyl concentrations were 1.5-fold higher in the Fe than in the Fe + Zn group. These indices did not differ in the Zn and Fe + Zn groups (Figure 3A and B). Activity

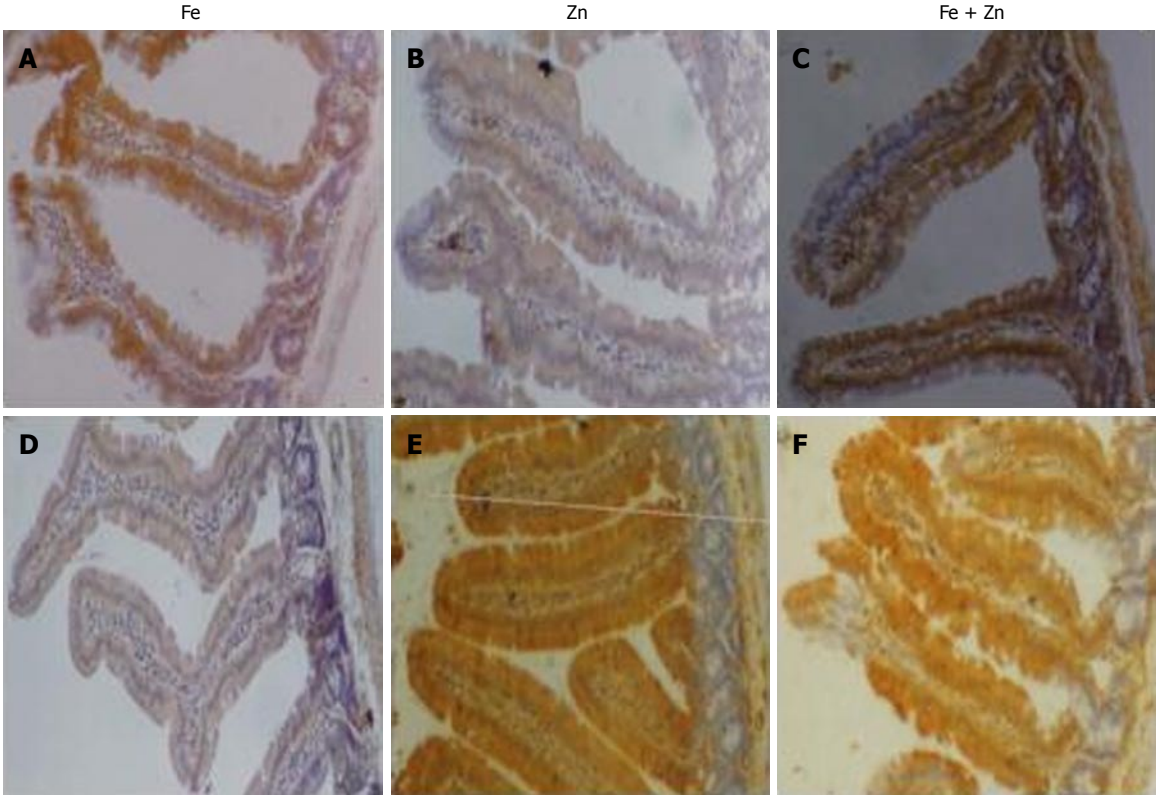

Figure 2 Zn reduces ferritin but enhances metallothionein abundance in the intestinal mucosa: A representative photomicrograph of the immunohistochemical localization of ferritin (top panel) and metallothionein (bottom panel) in the intestinal mucosa of Fe, Zn and Fe + Zn repleted groups. The staining intensity indicates the abundance of ferritin and metallothionein (× 250).

of the marker enzymes alkaline phosphatase and lys-ala-dipeptidyl aminopeptidase in the intestinal mucosa was about 20% lower in the Fe than in the Fe + Zn group. The activity did not differ between the Zn and Fe + Zn groups (Figure 3C and D).

Antioxidant status

Activity of intestinal SOD and glutathione peroxidase, except Mn-SOD, was significantly higher in the Fe than in the Zn and Fe + Zn groups. Catalase activity was significantly higher in the Fe and Fe + Zn groups compared to the Zn group. These enzyme activities did not differ between the Zn and Fe + Zn groups (Figure 4A-D), except for catalase activity. Glutathione concentration was significantly lower in the Fe than in the Zn and Fe + Zn groups. On the other hand, oxidized glutathione (GSSG) concentration was significantly higher in the Fe than in the Zn and Fe + Zn groups (Figure 5A and B).

Intestinal aconitase activity

Activity of cytosolic and mitochondrial aconitase was measured as an indicator of the cellular labile iron pool. Activity was higher in the Fe group, but lowered significantly in the Zn and Fe + Zn groups. Activity in the Fe + Zn group was higher than that in the Zn group (Figure 6A and B).

DISCUSSION

In the light of high prevalence of anemia, emerging incidence of zinc deficiency, and interactions during supplementation, we studied the interactive effects of iron and zinc repletion on oxidant/antioxidant status in combined deficient rats. Metabolic studies and supplementation trials suggest an antagonistic relationship between iron and zinc, in which zinc reduces the positive effects of iron supplementation and vice versa. For example, inorganic iron was found to compete for absorption with zinc when given to adults in solution in ratios > 2:1[2]. Zinc absorption in fasting pregnant Peruvian women treated with Fe or Fe + Zn was significantly reduced compared with non-metals supplemented women[23]; which indicates a post-absorptive effect of iron on zinc absorption. In women treated only with Fe, plasma zinc concentrations was also lower, compared with controls. Conversely, there were smaller improvements in hemoglobin and serum ferritin concentrations in Indonesian children treated both with Fe and Zn than in children treated with Fe alone[24]. Two recent studies on iron and zinc supplementation of Indonesian infants show that iron and zinc interactions occur when they are given as supplements[25,26]. In the study by Dijkhuizen *et al*, infants were treated with iron alone (10 mg/d), zinc alone (10 mg/d), both elements together (10 + 10 mg/d) and placebo from 4 to 10 mo of age. Supplementation significantly reduces the prevalence of iron deficiency anemia and zinc deficiency. Iron supplementation does not negatively affect plasma zinc concentration, and zinc supplementation does not increase the prevalence of anemia. However, combined iron and zinc supplementation is less efficacious than iron supplementation alone in reducing the prevalence

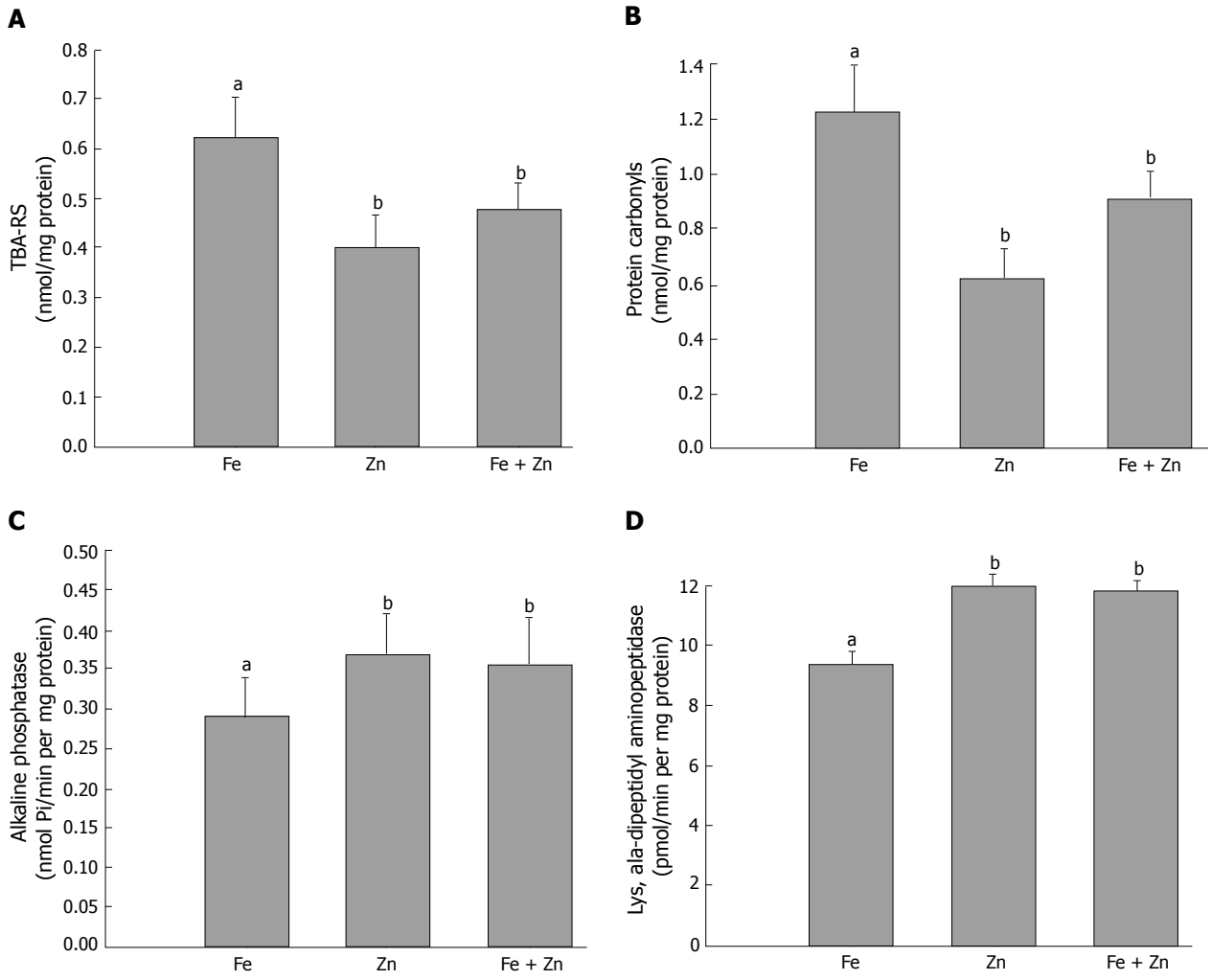

Figure 3 Iron increases intestinal oxidative stress and lowers functional integrity: Concentrations of thiobarbituric acid-reactive substances (TBA-RS) and protein carbonyls (**A** and **B**) as indicators of oxidative stress, and activities of alkaline phosphatase and lys-ala-dipeptidyl aminopeptidase (**C** and **D**), as markers of mucosal functional integrity, in the intestinal mucosa of iron and zinc deficient rats treated with iron and/or zinc. Fe: Iron administered group; Zn: Zinc administered group; Fe + Zn: Iron and zinc administered group; Number of animals: 7 in each group. Vertical columns and error bars represent mean and SD respectively; Bars with different superscripts are significantly different with $P < 0.05$ among groups.

of anemia (20% *vs* 38% reduction) and in increasing hemoglobin and plasma ferritin concentrations. In the study by Lind *et al*, infants received the same treatments, but from 6 to 12 mo of age. After supplementation, the Fe group had higher hemoglobin and serum ferritin than the Fe + Zn group; this fact indicates an effect of zinc on iron absorption. The Zn group had higher serum zinc than the placebo group, whereas this was not the case for the Fe and Fe + Zn groups, this fact suggests an effect of iron on zinc absorption. Thus, supplementation with Fe + Zn is less efficacious than single supplements alone in improving iron and zinc status, with evidence of negative interactions between iron and zinc when a combined supplement is given. However, the crucial question is whether such interactions between iron and zinc have any functional consequences for intestinal oxidative stress and functional integrity.

Both these nutrients are essential for growth, and in populations in which deficiencies of these minerals co-exist, stunting of growth has been reported[27]. Depletion of both iron and zinc in the diet has a significant impact on the growth of rats, and causes a marked reduction in iron and zinc status. Thus, the model developed in the present study had characteristic clinical and sub-clinical manifestations of iron and zinc deficiency, respectively. Although several studies have examined the effect of iron supplementation on zinc absorption, few have considered the effect of zinc supplementation on iron absorption. The molar ratio of iron and zinc seems to be critical in determining the interactive effects. One study has shown that zinc inhibited iron absorption when the zinc-iron ratio was 1.14:1 (1:1 molar ratio), but not when it was 0.36:1 (0.4:1 molar ratio)[28]. Another study has shown that a zinc-iron ratio of 5:1 significantly reduced iron absorption from an aqueous solution, but did not affect heme iron absorption from a hamburger meal in humans[29]. In the present study, we used a 1:1 molar ratio for repletion of iron- and zinc-deficient rats.

Several possible sites of interaction during transfer from the apical membrane into the intestinal cytosolic compartment, and from the basolateral membrane into plasma have been suggested by Fairweather-Tait[30].

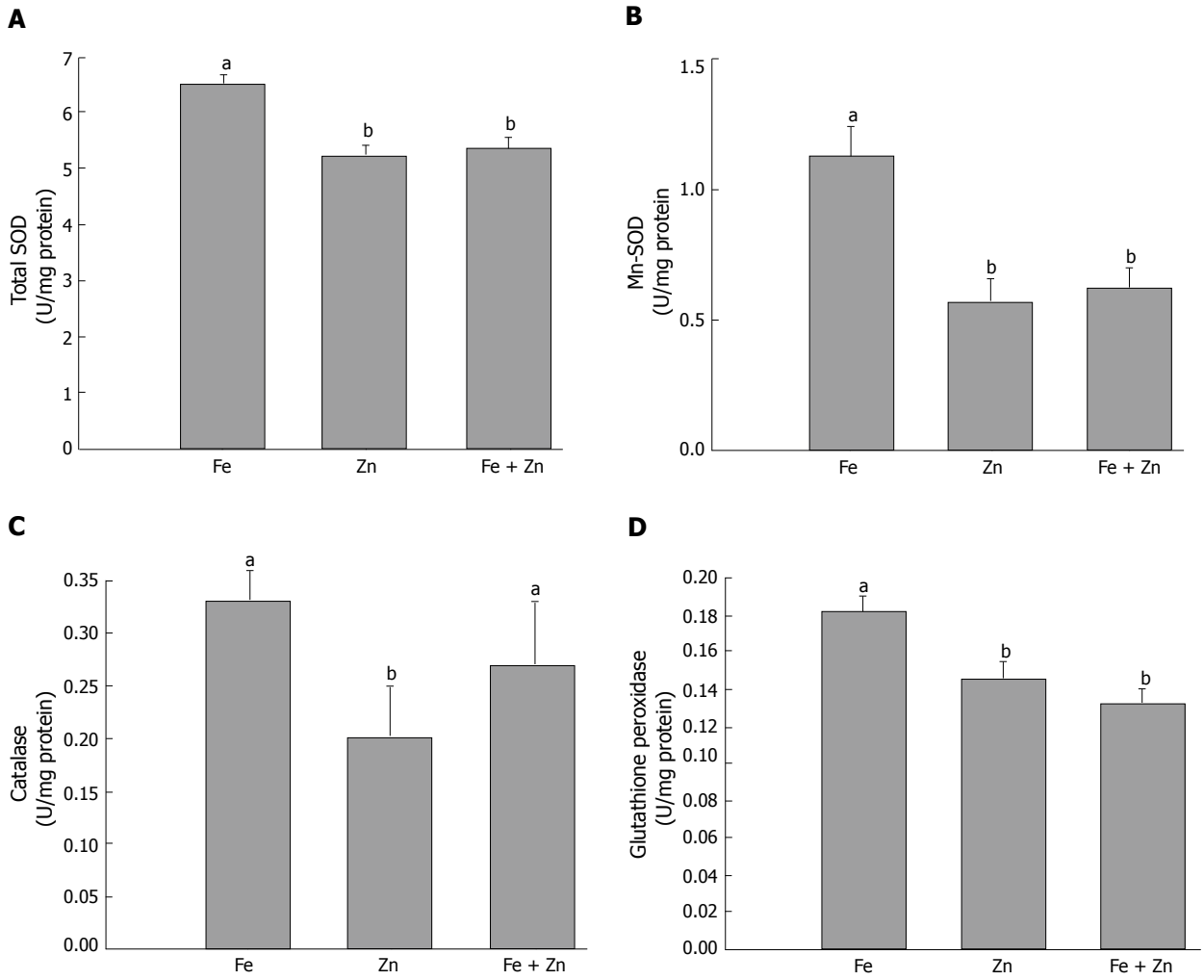

Figure 4 Changes in antioxidant enzyme activities due to iron and zinc repletion: Activity levels of superoxide dismutase (SOD, **A** and **B**), catalase (**C**) and glutathione peroxidase (Gpx, **D**) in the small intestinal mucosa of rats during repletion with iron and/or zinc. Total SOD activity indicates the activity of Cu, Zn-SOD and Mn-SOD. Fe: Iron administered group; Zn: Zinc administered group; Fe + Zn: Iron and zinc administered group; Number of animals: 8 in each group. Vertical columns and error bars represent mean and SD respectively; bars with different superscripts are significantly different with $P < 0.05$.

The possibility of iron and zinc inhibiting each other's intestinal uptake through competition for a common pathway, has been studied by measuring ferritin and metallothionein concentrations, and aconitase activity in the site of absorption. Intestinal uptake of iron and zinc was significantly reduced in the presence of the other metal during repletion in rats with combined deficiencies of iron and zinc. This may have been due to increased competition between iron and zinc for the ligands/transporters at the site of absorption. Reduced uptake of ^{55}Fe or ^{65}Zn in the Fe + Zn group compared to the Fe or Zn groups provides clear evidence for their antagonistic interaction during repletion. Reduced plasma and liver iron and zinc concentrations in the Fe + Zn group compared to individual supplementation groups suggests that iron and zinc interactions affect not only the uptake, but also retention of these minerals. Thus, this study clearly demonstrates that a combined supplement is less efficacious than a single supplement in improving iron or zinc status.

Another intriguing possible site of interaction between iron and zinc is the duodenal transport protein divalent cation transporter 1 (DCT-1). DCT-1 appears to be a key transporter that is involved in iron absorption, but can also transport many other metals, including zinc[31]. It is possible that iron and zinc can inhibit each other's absorption by competing for DCT-1, and their effects are expected to be most noticeable when one metal is in relative excess compared with the other, or when both deficiencies co-exist. Although we have not studied DCT-1 expression, the influence of iron and zinc on their responsive proteins clearly suggests that both interact at the site of absorption during concurrent repletion.

The intestine is vulnerable to oxidative damage during iron and/or zinc depletion and repletion. Incessant pulses of iron during repletion leave excess unabsorbed iron in the intestinal mucosa, which is a potential pro-oxidant. Moreover, electron paramagnetic resonance spectroscopy in rats has shown that oral iron therapy with ferrous sulfate results in iron-mediated oxidative stress, through hydroxyl radicals in the small intestine. This stress-stimuli results in a decrease in cell turnover, shortening of microvillus height, and partial or complete erosion of the microvilli in the duodenum[32-34]. On the

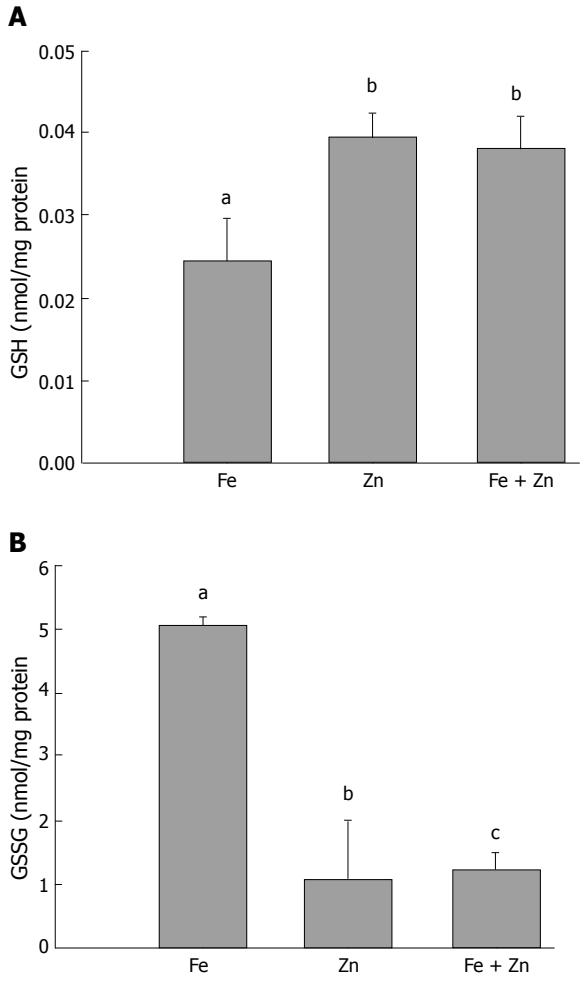

Figure 5 Altered intestinal mucosal redox status: Changes in the levels of GSH (Panel **A**) and GSSG (Panel **B**) in the intestinal mucosa at the end of repletion for 2 wk with iron and/or zinc. Vertical columns and error bars represent mean and SD respectively; bars with different superscripts are significantly different with $P < 0.05$.

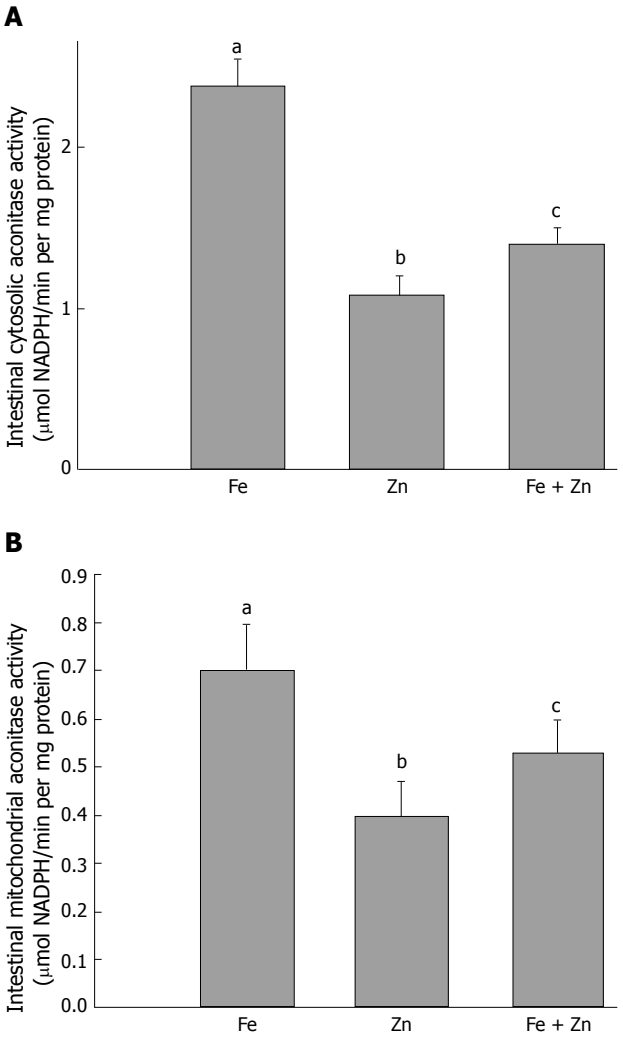

Figure 6 Intestinal aconitase activity in response to iron and/or zinc repletion: Response of intestinal cytosolic (Panel **A**) and mitochondrial (Panel **B**) aconitase to intestinal iron content and as a functional indicator of oxidative stress after 2 wk of oral iron and/or zinc administration. Fe: Iron administered group; Zn: Zinc administered group; Fe + Zn: Iron and zinc administered group. Number of animals :is 8 in each group. Vertical columns and error bars represent mean and SD respectively; bars with different superscripts are significantly different with $P < 0.05$.

other hand, zinc deficiency impairs intestinal antioxidant capacity by lowering the expression of metallothionein, an effective scavenger of hydroxyl radicals that can play a major role in the development of oxidative damage. In an earlier study, we have shown increased hydroxyl radical production, associated decrease in turnover of intestinal epithelial cells, and compromised functional integrity of the mucosa, when iron-deficient rats were repleted with 8.0 mg Fe[12]. This dose may be relatively high and could have produced structural and functional impairment at the site of absorption. To reduce these effects, we used 4.0 mg Fe for repletion and maintained a 1:1 molar ratio of iron and zinc in the present study. Iron repletion resulted in significantly higher levels of peroxidation products, i.e., TBARS and protein carbonyls in the intestinal mucosa, even with lower doses of iron. This also decreased the alkaline phosphatase and dipeptidase activity, which indicates compromised functional integrity. Although zinc negatively affected iron uptake, concurrent repletion of iron and zinc significantly reduced the oxidative damage and improved the functional integrity. The reason for the beneficial effects with co-administration of iron and zinc may be due to the changes in antioxidant balance. We have observed increased localization of metallothionein in the intestinal mucosa during zinc repletion. These findings support the view that zinc *per se* can act at various levels and exert its antioxidant effect. Incorporation of zinc along with iron in supplements seems to be efficacious in improving the antioxidant capacity.

To determine the role of antioxidant defense enzymes in reducing iron-induced oxidative damage, activity of SOD, catalase and glutathione peroxidase were determined. Cu, Zn-SOD was more active in the Fe group compared to that in the Zn and Fe + Zn groups. On the other hand, Mn-SOD activity was similar in all three groups. This indicates that the formation of superoxide anion and its conversion to hydrogen peroxide is greater in the cytosolic compartment than in the mitochondria. The other antioxidant enzyme that showed a difference in the Fe and Zn + Fe groups was glutathione peroxidase (Gpx), which implies that reduction of peroxides to water was more prevalent in the Fe than in the Fe + Zn group.

No increase was observed in the catalase activity of iron-treated animals, which suggests that hydrogen peroxide conversion to water was similar among all groups, but was compensated by an increase in Gpx in the Fe group. This increase in Gpx is indicative of excess formation of organic peroxides in the Fe group, which may have enhanced oxidative stress. In a recent study, we have shown that zinc *per se* can reduce iron-mediated production of hydroxyl radicals and thereby protect against oxidative stress[8]. Hence, concurrent administration of iron and zinc or zinc alone substantially enhances the intestinal non-enzymatic antioxidant capacity. In addition, we have observed in zinc-supplemented groups, increased intestinal metallothionein concentrations, which has been reported to be more effective than GSH on a molar basis for preventing oxidative damage of various cellular components[35]. Quenching of hydroxyl radicals by sulfhydryl groups in metallothionein, releasing zinc and its subsequent uptake by the membranes can protect both cellular components and membranes against oxidative damage[36,37]. Thus, zinc-induced metallothionein not only reduces the oxidative stress, but also improves the functional integrity of the mucosa.

Control of iron uptake and storage through regulation of transferrin receptor and ferritin proteins represents an important avenue through which cellular iron homeostasis is modulated and maintained[38,39]. The expression of ferritin and transferrin receptor is linked to iron status through the action of two iron-regulated RNA-binding proteins, iron regulatory proteins, IRP1 and IRP2. Iron regulates the RNA-binding function of IRP1 and IRP2 through fundamentally different mechanisms[40,41]. For IRP1, which is a bifunctional protein, iron inhibits RNA-binding activity by promoting assembly of an iron-sulfur cluster in the binding protein, thereby converting it to cytosolic aconitase[42]. Walter *et al*[43] has suggested that iron deficiency can induce the IRP-mediated cellular iron signaling pathway, which leads to enhanced intracellular iron levels. Therefore, we assessed the activity of cytosolic and mitochondrial aconitase. Cytosolic aconitase activity was found to be suppressed in the presence of zinc in the Fe + Zn group compared to that in the Fe group. A decrease in cytosolic aconitase activity implies a decrease in cellular iron availability in the presence of zinc, or inhibition of aconitase *per se* by zinc. It has been shown that zinc competitively inhibits duodenal cytosolic aconitase activity[22]. Reduced ferritin protein in the intestinal mucosa of the Fe + Zn group when compared to that of the Fe group supports the decrease in cytosolic aconitase activity in the Fe + Zn group. We also observed a decrease in mitochondrial aconitase activity in the Fe + Zn compared to the Fe group. This may have been due to a decrease in citrate availability or post-transcriptional regulation by IRP1, as reported by Chen *et al*[44]. Nevertheless, zinc seems to act as a buffer against free-radical damage invoked by the presence of free iron. The presence of zinc during supplementation had a significant effect on the levels of various antioxidants, including GSH and metallothionein.

Thus, this study clearly demonstrates that the interactions between iron and zinc during absorption in iron- and zinc-deficient rats are mutually antagonistic. Competition of iron and zinc for common transporters (DMT1) at the site of absorption results in reduced uptake of these minerals during concurrent administration. The presence of zinc during iron administration also influences aconitase activity, ferritin expression and thereby the labile iron pool. Furthermore, zinc induction of metallothionein and scavenging of hydroxyl radicals helps to control iron-mediated oxidative stress.

COMMENTS

Iron deficiency is the single most common nutritional disorder worldwide and the main cause of anemia in infancy, childhood and pregnancy. It is prevalent in most of the developing world, where it coexists with other micronutrient deficiencies such as zinc, vitamin A and folate. The exact prevalence of zinc deficiency is not known, but it is estimated that the magnitude might not be too different from that for iron deficiency. This is probably because the diet of populations in the developing world is based mainly on foodstuffs that have low iron and zinc concentrations, and with a poor bioavailability of these minerals. Combined supplementation with iron and zinc in target populations may be effective in preventing deficiencies of these micronutrients, but knowledge of their potential interactions when given together is inadequate.

Research frontiers

Combined supplementation with both micronutrients is one strategy that can be used to improve the iron and zinc status of a population. However, there is concern about the negative interactions between these two minerals. Studies performed in humans have shown an inhibitory effect of zinc on iron absorption, but it is not well established whether this interaction depends on the absolute amount of iron and zinc in the supplement and/or on the molar ratio between these two minerals, or on nutritional status. This information could help design rational guidelines for iron and zinc supplementation programs. A review of the randomized trials that have assessed the effects of iron and zinc supplementation on iron and zinc status shows that zinc supplementation alone does not appear to have a clinically important negative effect on iron status. However, when zinc is given with iron, iron indicators do not improve as greatly as when iron is given alone. In most of the studies, iron supplementation did not affect the biochemical status of zinc, but the data are not clear regarding morbidity outcomes. Although some trials have shown that joint iron and zinc supplementation has less effect on biochemical or functional outcomes than supplementation with either mineral alone, there is no strong evidence to discourage joint supplementation. Supplementation programs that provide iron and zinc together are an efficient way to provide both micronutrients, provided the benefits of individual supplementation are not lost. Further research is needed before health policies on joint supplementation programs can be established.

Innovations and breakthroughs

Oral iron supplementation is a widely used practice to correct iron-deficiency anemia. Exposure of iron-deficient intestine to large doses of iron is known to induce oxidative damage, which leads to loss of functional integrity and reduced mucosal cell turnover. Intestinal conditioning with anti-oxidants during iron administration has been shown to suppress iron-induced oxidative damage. Zinc is known to protect cells from peroxidative damage by inducing metallothionein and maintaining sulfhydryl group stability. Nevertheless, co-administration of iron and zinc may antagonize each other with respect to absorption. In the present study, we showed that although combined supplementation of iron and zinc marginally inhibits iron uptake, it significantly attenuates oxidative stress by induction of metallothionein and elevation of GSH level. Furthermore, the presence of zinc *in situ* reduced the iron-induced hydroxyl radical production in the intestinal mucosa, as assessed by electron paramagnetic resonance spectroscopy. These results strongly suggest a protective role for zinc on iron-induced oxidative stress, which might have implications in anemia control programs.

Applications

Supplementation with multiple micronutrients is an appealing strategy for the prevention and treatment of anemia and common morbidities that affect women and young children. However, drawing definitive conclusions regarding the

potential benefit or harm of joint supplementation, based on a variety of study designs, target populations and outcome measures, has proven challenging.

Terminology

Nutrient-nutrient interactions: Although the term interaction denotes a bidirectional effect, many interactions are unidirectional, i.e., one nutrient affects the biological disposition of another, which remains more or less passive. Bidirectional interactions are most common among nutrients with similar physicochemical properties and that share a common mechanism of absorption or metabolism. Some uni- or bidirectional interactions are affected by the presence of a third dietary constituent. Nutrient interactions are not usually additive. From the physiological standpoint, nutrient interactions can occur at several different levels: (1) In the diet. The mode of preparation of diets may be as important as their composition in determining nutrient interactions. For example, cooking in an alkaline medium may decrease the interaction between ascorbic acid and iron by destroying the vitamin; (2) In the intestinal lumen. Interactions at this level have received the most attention, because they determine the true availability of a nutrient for translocation through the enterocytes. Most luminal interactions consist of direct nutrient-nutrient interactions, but certain nutrients can indirectly affect the absorption of others by modifying gastrointestinal physiological activities. For example, certain dietary fibers can stimulate gastrointestinal hormone secretion or inhibit micellar formation, thus indirectly affecting nutrient absorption (Table 2); (3) In the post-absorptive phase. Many interactions take place after the process of absorption has been completed. These interactions may be in the form of physiological synergism, such as the effect of vitamin A and zinc on the visual process, or between vitamin A and iron mobilization. Conversely, negative interactions may affect circulating or storage levels of nutrients.

Peer review

In this study, the authors investigated the nature of interactions between iron and zinc, and their consequences on intestinal oxidant-antioxidant balance. They demonstrated that the interactions between iron and zinc during absorption in iron- and zinc-deficient rats were antagonistic. They demonstrated that the antioxidant status was significantly lower in the Fe than in the Zn and Fe + Zn groups, whereas the aconitase activity was higher in the Fe than in the Zn and Fe + Zn groups. In conclusion, they reported that the competition of iron and zinc for common transporters such as DCT-1, at the site of absorption, resulted in reduced uptake of Fe and Zn during concurrent administration. Moreover, they suggested that zinc induction of metallothionein and scavenging of hydroxyl radicals assisted in controlling iron-mediated oxidative stress.

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BASIC RESEARCH

Silencing SMYD3 in hepatoma demethylates RIZI promoter induces apoptosis and inhibits cell proliferation and migration

Li-Bo Chen, Jun-Yao Xu, Zhen Yang, Guo-Bin Wang

Li-Bo Chen, Guo-Bin Wang, Hepatobiliary center, Union Hospital of Huazhong University of Science & Technology, Wuhan 430022, Hubei province, China
Jun-Yao Xu, Department of General Surgery, The Second Affiliated Hospital of Sun Yat-seu University, Guangzhou 510120, Guangdong province, China
Zhen Yang, Department of integrated surgery, Tongji Hospital of Huazhong University of Science & Technology, Wuhan 430030, Hubei province, China
Supported by National Natural Science Foundation of China, No 30200273 & 30672067
Correspondence to: Li-Bo Chen, Hepatobiliary center, Union Hospital of Huazhong University of Science and Technology, Wuhan 430022, Hubei province, China. libo_chen@hotmail.com
Telephone: +86-27-85351623 Fax: +86-27-85776343
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Abstract

AIM: To investigate the role of SMYD3 in hepatocellular carcinoma (HCC) development and progression and to verify whether its regulation activity was through RIZ1 inactivation.

METHODS: Expression of SMYD3 in HCC cell lines and tissues were measured; silencing of SMYD3 by RNA interference (RNAi) was effectuated, hepatoma cell proliferation, migration and apoptosis were tested, with RIZ1 CpG promoter methylation, and corresponding mRNA expression were investigated.

RESULTS: SMYD3 over-expression in HCC was associated with RIZ1 hypermethylation and mRNA down-expression. Suppression of SMYD3 expression de-methylated RIZ1 CpG promoter ($P < 0.01$) and increased RIZ1 mRNA expression ($P < 0.01$). Consequently, SMYD3 down-expression with RIZ1 de-methylation strongly inhibited hepatoma cell growth (MTT inhibitory rates: Pgenesil-1-s1 60.95% ± 7.97%, Pgenesil-1-s2 72.14% ± 9.68% *vs* Pgenesil-1-hk 6.89% ± 4.12%, $P < 0.01$) and migration (Pgenesil-1-s1 4.24% ± 1.58%, Pgenesil-1-s1 4.87% ± 0.73% *vs* Pgenesil-1 19.03% ± 4.63%, Pgenesil-1-hk 19.95% ± 5.21%, $P < 0.01$) and induced apoptosis (FCM subG1 phase Pgenesil-1-s1 19.07% ± 1.78%, Pgenesil-1-s2 17.68% ± 2.36% *vs* Pgenesil-1 0.47% ± 0.12%, Pgenesil-1-hk 1.46% ± 0.28%, $P < 0.01$. TUNEL-positive cells: Pgenesil-1-s1 40.24% ± 5.18%, Pgenesil-1-s2 38.48% ± 4.65% *vs* Pgenesil-1 2.18% ± 1.34%, Pgenesil-1-hk 2.84% ± 1.22%, $P < 0.01$) in HepG2 cells.

CONCLUSION: These results demonstrate that SMYD3 plays a critical role in the carcinogenesis and progression of HCC. The proliferation, migration induction and apoptosis inhibition activities of SMYD3 may be mediated through RIZ1 CpG promoter hypermethylation.

Key words: SMYD3; Hepatocellular carcinoma; Retinoblastoma protein-interacting zinc finger gene; Histone methyltransferase; DNA methylation

Chen LB, Xu JY, Yang Z, Wang GB. Silencing SMYD3 in hepatoma demethylates RIZI promoter induces apoptosis and inhibits cell proliferation and migration. *World J Gastroenterol* 2007; 13(43): 5718-5724

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, generally long-term survival is disappointing. Epigenetics, defined as heritable changes in gene expression that are not coded in the DNA sequence itself, is increasingly linked with tumorigenesis[1,2]. It is well established that DNA methylation, nucleosomal histone post-translational modifications such as methylation, acetylation/deacetylation, phosphorylation ADP-ribosylation, and ubiquitination, and other epigenetic patterns are central to proper gene expression. Increasing evidence shows that disrupting epigenetic patterns can induce carcinogenesis or affect the outcome of cancer[3,4]. Among these, histone methylation is considered to be critical for transcriptional regulation and seems to play an important role in tumor epigenetic modification. Recently, SMYD3 was identified and characterized to specifically methylate histone H3 at lysine 4 (K4), and this activity is indeed dependent on an intact SET domain, an evolutionarily conserved protein module shown to facilitate histone methyltransferase activity[5]. Expression of SMYD3 was frequently enhanced in colorectal carcinoma (CRC), breast cancer tissue, as well as HCC, and elevated *SMYD3* expression was involved in the growth of CRC, breast cancer and HCC cells[5-7]. Although transcriptional activation of downstream genes including *Nkx2.8* and *WNT10B* gene has been reported[5,6], the effect of SMYD3 on HCC development and the underlining mechanism remains unclear.

Inactivation of tumor suppressive genes (TSGs) plays an important role in carcinogenesis. RIZ1, a typical

TSG with H3-K9 methyltransferase activity, was shown to lose its expression and tumor-suppressing activity in many types of human tumors including HCC[8], CRC[9], breast cancer[10], prostate cancer[11] and gastric cancer[12]. Adenovirus-mediated RIZ1 expression causes G2-M cell cycle arrest and/or apoptosis in breast cancer, liver cancer, and microsatellite instability-positive colon cancer cells. Adenovirus RIZ1 can also inhibit growth of colon cancer xenografts[13]. Previous data suggest that the RIZ locus is a target of frequent deletion in HCC, but a more common way of RIZ inactivation in HCC may not involve mutations that alter peptide sequences, but by CpG promoter hypermethylation[14].

Therefore, we sought to explore whether targeting of SMYD3 expression can inhibit the development of HCC, and determine whether the biological function of SMYD3 in HCC development and progression is mediated through RIZ1 inactivation. We found SMYD3 was significantly overexpressed in HCC cell lines, but not in normal hepatocellular cells and peri-cancerous tissues. Silencing of SMYD3 by RNAi remarkably inhibited HCC cell proliferation and migration, and induced apoptosis *in vitro*. Furthermore, RIZ1 was under-expressed in HCC cells with its promoter hypermethylated, while inhibition of SMYD3 demethylated RIZ1 and recovered its expression in HCC cells. Our results indicated that SMYD3 plays pro-oncological function in HCC development and progression; thus the activity of SMYD3 in HCC may be partly through its activity on RIZ1 promoter hypermethylation and RIZ1 inactivity.

MATERIALS AND METHODS

Cell lines

HCC cell lines HepG2 and Hep3B were purchased from the American Type Culture Collection (Rockville, MD). HCC cell line SMMC-7721 and liver cell line L-02 were generous gifts from Dr. Danhui Weng (HUST, China). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco-BRL).

RT-PCR

Total RNA was extracted using Trizol reagent (Takara, Tokyo, Japan). RT-PCR was performed with the Advantage RT-PCR kit (Takara), according to the manufacturer's protocol. PCR setting: Initial denaturation at 94℃ for 3 min before 18 cycles (for *GAPDH*) or 30 cycles (for *SMYD3* and *RIZ1*) at 94℃ for 30 s, 50℃ for 45 s and 72℃ for 60 s. The sets of primers are listed in Supplementary Information Table S1.

shRNA construction and transfection

The Pgenesil-1 vector containing the U6 promoter region was purchased from Genesil Biotechnology Corporation (Wuhan, China). Plasmids expressing siRNAs were prepared by cloning double-stranded oligonucleotides into the Pgenesil-1 vector. The sets of primers are listed in Supplementary Information Table S1. Positive clones were identified by restriction digestion and confirmed by sequencing. The resulting plasmids were designated as Pgenesil-1-s1, Pgenesil-1-s2 and negative control Pgenesil-1-hk. Transfection was performed by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol.

Western blot analysis

Proteins were prepared by homogenization of cells in lysis buffer (10 mmol/L Tris-HCl, pH 8.0; 140 mmol/L NaCl; 5 mmol/L EDTA; 0.25 g/L NaN_3; 10 g/L Triton X-100; 10 g/L deoxycholate; 1 g/L SDS; 0.5 mmol/L PMSF; 1 g/L leupeptin; 1 g/L aprotinin). Protein concentration was determined by the Lowry method (Bio-Rad, Hercules, CA). Proteins were resolved (30 μg per lane) in 120 g/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was transblotted onto a PVDF membrane (Amersham, Arlington Heights, IL). Membranes were blocked in 50 mL/L non-fat milk overnight and subsequently incubated with 1:2000 rabbit polyclonal anti-SMYD3 antibody (a generous gift from Dr. Ryuji Hamamoto of the University of Tokyo, Tokyo, Japan), or anti-β-actin (1:5000 dilution, Sigma, St. Louis, MO) for 1 h and then with secondary anti-rabbit IgG-horseradish-peroxidase antibody for 1 h at room temperature. Immunoreactive proteins were visualized by means of enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) and exposure to autoradiographic film.

Proliferation assay of HepG2 cell

MTT experiments were carried out using the cell proliferation kit (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's manual. Briefly, stable transfected and parental HepG2 cells were seeded at a density of 5×10^3 cells/well in 96-well plates and allowed to grow for 48 h and then 20 μL 5 g/L MTT was added. After incubation for 4 h, DMSO 200 g/L was added to each well to dissolve crystals. After 5 min incubation at 37℃, absorbance was measured at 570 nm. Assays were performed in triplicate.

Migration assay of HepG2 cell

This assay for the invasiveness of cells was based on the principle of Boyden chamber (BD Biosciences, San Diego, CA) and performed according to the manufacturer's protocol. Briefly, the top compartment was prepared by coating the filter with diluted Matrigel and incubated for 30 min. A suspension of 1×10^4 cells in serum-free medium was inoculated in the upper chamber, and conditioned media obtained from NIH3T3 cells was placed in the lower compartment of the chamber, as a chemoattractant. After 24 h incubation, noninvasive cells were removed with a cotton swab. The cells which migrated through the filter and adhered to the lower surface of the filter were fixed with methanol, stained with hematoxylin, and counted manually in 5 randomly selected microscopic fields. Assays were performed in triplicate. The data were reported as the percentage of cells successfully passing through the Matrigel and filter relative to those migrating through the control filter.

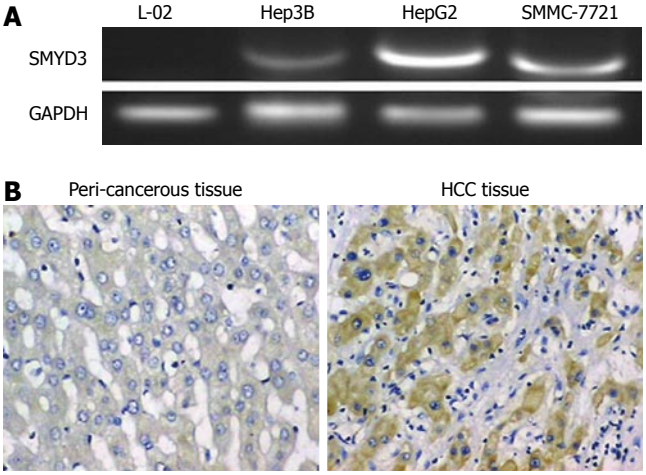

Figure 1 Enhanced expression of SMYD3 in HCC. **A**: The expression of SMYD3 genes was determined by RT-PCR and GAPDH served as an internal control; **B**: Representative images of immunohistochemical staining of accumulated SMYD3 protein in HCC tissue, but not in peri-cancerous tissue.

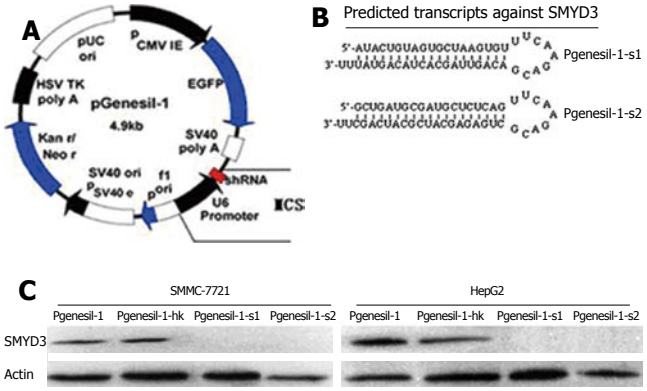

Figure 2 RNAi specifically inhibits SMYD3 expression in HCC cell lines. **A**: Schematic drawing of the Pgenesil-1 vector; **B**: The predicted secondary structures of the Pgenesil-1-s1 and Pgenesil-1-s2 transcripts target SMYD3 are shown; **C**: 48h after transfection, Western blot analysis showed the inhibitory effect of plasmids expressing SMYD3 shRNAs in HepG2 and SMMC-7721 cells.

FCM analysis

HepG2 cells, 1×10^6, were seeded in 60-mm dishes and transfected with SMYD3-RNAi-plasmids at 60%-80% confluence. Then, 48 h after transfection, cells were deprived of serum for 36 h. Afterwards, cells were harvested, fixed in 700 mL/L ethanol and washed with 1 × PBS and suspended in 50 mg/L propidium iodide. DNA content was determined by flow cytometry using a Becton-Dickinson FACSCalibur (Becton Dickinson, Bedford, MA). Results were analyzed by ModFit LT2.0 and Cellquest software. Assays were performed in triplicate.

TUNEL assay

Apoptotic cells were identified with the *In Situ* Cell Death Detection kit (Roche Applied Science, Shanghai, China) using the protocol recommended by the manufacturer. In brief, HepG2 cells were grown on coverslips. The next day, cells were transfected with SMYD3-RNAi-plasmids. At 48 h after transfection, cells were deprived of serum for 36 h. Coverslips with adherent cells were fixed in 40 g/L paraformaldehyde for 1 h at room temperature and permeabilized with 1 g/L Triton X-100 for 2 min on ice. DNA fragments were labeled with the TdT-mediated dUTP nick end labeling (TUNEL) reaction mixture for 60 min at 37℃ in a humidified atmosphere in the dark. Diaminobenzidine was used to mark the apoptotic cells (brown staining). Cells were counted manually in six randomly selected microscopic fields for each sample. The apoptosis index was defined by the percentage of apoptotic cells among the total cells of each sample.

RIZ1 promoter methylation specific PCR assay

Methylation Specific PCR (MSP) assay was performed according to the procedure described by Fang *et al*[15]. In brief, 1 μg of the genomic DNA was modified by sodium bisulfide using the CpGenome DNA Modification Kit (Intergen, Purchase, NY) in accordance with the manufacturer's instructions. Modified DNA was amplified by two different primer pairs specific to the unmethylated (U) and methylated (M) *RIZ1* sequences, respectively. The sets of primers are listed in Supplementary Information Table S1. The PCR amplification was performed for a total of 45 cycles with an annealing temperature of 68℃ and 60℃ for M-sequences and U-sequences, respectively. The PCR products were then analyzed using a 35 g/L agarose gel.

Statistical analysis

SPSS 13.0 was used to analyze the data. Statistical significance was assessed by comparing mean ± SD with Student's *t* test for independent groups. $P < 0.05$ was considered statistically significant.

RESULTS

Enhanced SMYD3 expression in HCC cell lines

To determine whether SMYD3 gene is overexpressed in HCC cell lines, we compared the level of SMYD3 gene expression in normal human hepatocellular cell line L-02 to that of three HCC cell lines. RT-PCR analysis revealed that SMYD3 was overexpressed in all HCC cells, but absent in L-02 cells (Figure 1). These data indicate that enhanced SMYD3 expression is involved in a majority of HCC.

RNAi specifically inhibits SMYD3 expression in HCC cell lines

To inhibit SMYD3 expression in HCC cells, we used a DNA-based siRNA method (Figure 2A). Two siRNAs targeting different 21 sequences of human SMYD3 were cloned into Pgensil-1 vector to express RNA, which is expected to fold back to form a hairpin loop structure after being transcribed. The hairpin dsRNA can then be further cleaved by Dicer to generate a 21-nucleotide siRNA, the active form for the RNAi effect (Figure 2B). We used HepG2 and SMMC-7721 cells, which overexpress SMYD3 abundantly. Forty eight hours after transfection, Pgenesil-1-s1 and Pgenesil-1-s2 markedly knocked down SMYD3 expression in these cells, as determined by Western blot analysis. The specificity of RNAi targeting SMYD3 was shown by transfection with Pgenesil-1-hk which had no effect on SMYD3 expression (Figure 2C).

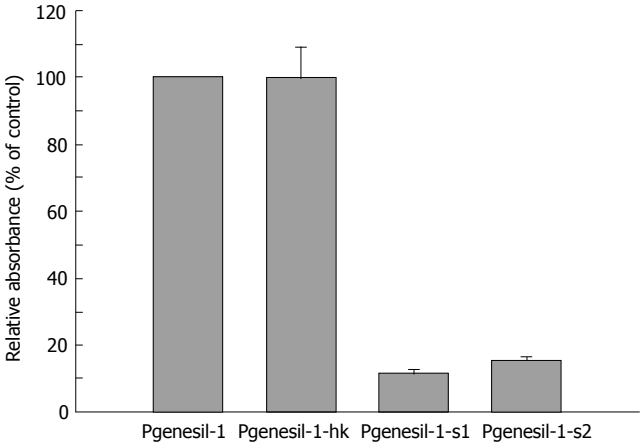

Figure 3 Inhibition of SMYD3 reduces hepatoma cell proliferation (MTT assays).

Inhibition of SMYD3 reduces hepatoma cell proliferation

MTT assay was used to examine the inhibitory effect of RNAi against SMYD3 on cell growth in HepG2 cells. Suppression of SMYD3 expression significantly inhibited cell growth compared to the cells transfected with control plasmid Pgenesil-1. Pgenesil-1-hk, which did not suppress expression of SMYD3, was shown to have little inhibitory effect on growth of HepG2 cells, compared to Pgenesil-1 (Figure 3). The growth inhibitory effect of the plasmids was consistent with their gene silencing effect. Therefore, SMYD3 RNAi significantly suppressed the growth of HeG2 cells *in vitro*, indicating that SMYD3 may be involved in the regulation of cell proliferation.

Inhibition of SMYD3 reduces hepatoma cell migration

The HepG2 cell line has been characterized as a highly invasive hepatocellular cancer cell line. To determine whether SMYD3 gene knockdown by RNAi could reduce its invasive potential, an *in vitro* invasion assay was performed. As shown in Figure 4, HepG2 cells were greatly deprived of invasiveness by depletion of SMYD3 gene expression. The percentage of invasive cells was decreased by 3-4 fold in Pgenesil-1-s1 and Pgenesil-1-s2 treated groups, compared to Pgenesil-1 treated groups. No significant decrease of invasive cells was observed in Pgenesil-1-hk treated cells. These results demonstrate that SMYD3 not only plays a pivotal role in the process of development, but it also is involved in tumor cell migration.

Inhibition of SMYD3 promotes apoptosis in hepatoma cells

Flow cytometry analysis showed that after 48 h of transfection and 36 h serum deprivation, the number of cells in the sub G1 phase was 19.07 ± 1.78% and 17.68 ± 2.36% in HepG2 cells transfected with Pgenesil-1-s1 and Pgenesil-1-s2, respectively, while only 0.47% ± 0.12% and 1.46% ± 0.28% cells were observed in HepG2 cells transfected with Pgenesil-1 and Pgenesil-1-hk, respectively (Figure 5A). These results possibly suggest the induction of apoptosis. To confirm whether silencing of SMYD3 can induce apoptosis in HCC cells following serum deprivation, TUNEL assay was performed. The number

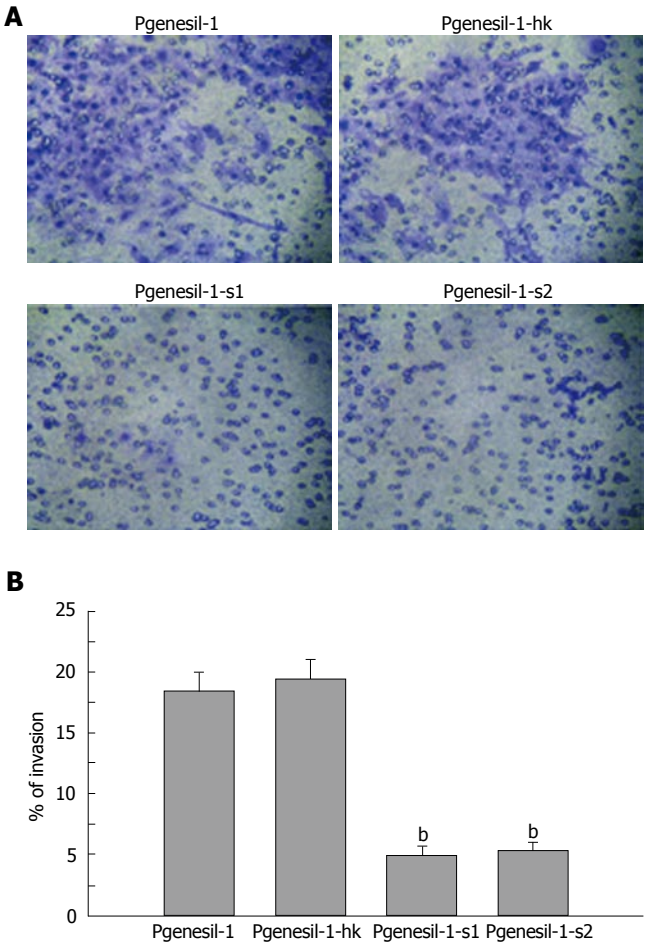

Figure 4 Inhibition of SMYD3 reduces hepatoma cell migration. **A**: Representative cells (blue) invade through Matrigel and membrane; **B**: The percentage of cells successfully passing through the matrigel and membrane ($P < 0.01$).

of TUNEL-positive cells was 40.24% ± 5.18% and 38.48% ± 4.65% in HepG2 cells transfected with Pgenesil-1-s1 and Pgenesil-1-s2, respectively. Only 2.18% ± 1.34% and 2.84% ± 1.22% were TUNEL positive in control cells transfected with Pgenesil-1 and Pgenesil-1-hk, $P < 0.01$, respectively (Figure 5B). These results indicate that inhibition of SMYD3 by RNAi significantly promoted apoptosis in HepG2 cells following serum deprivation.

Inhibition of SMYD3 de-methylates RIZ1 promoter CpG islands and re-expresses RIZ1 in hepatoma cells

Through MSP and RT-PCR, we observed that the RIZ1 promoter was totally methylated with the lack expression of RIZ1 mRNA in HepG2 cells. After inhibition of SMYD3 by Pgenesil-1-s1, the RIZ1 promoter was found partial methylated in HepG2 cells, consistent with the change of RIZ1 mRNA (Figure 6). These results suggest that the activity of SMYD3 may be regulated through RIZ1 CpG promoter hypermethylation and RIZ1 inactivation.

DISCUSSION

Among epigenetic regulatory ways, histone methylation, perhaps more than any other form of modification, has demonstrated the power of modifications over DNA-

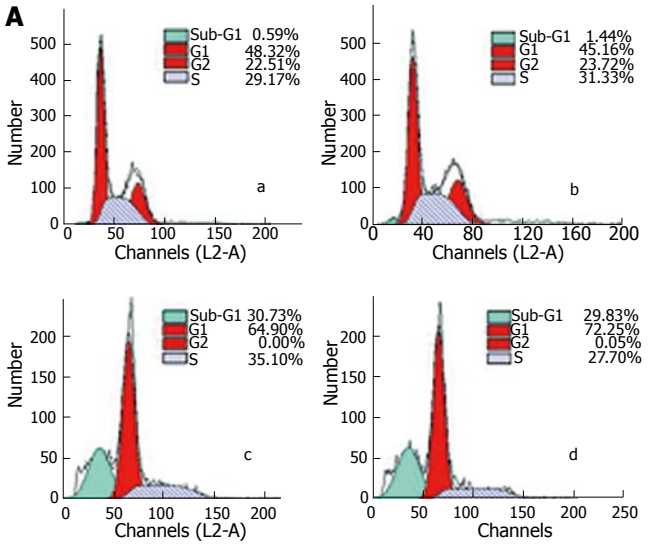

Figure 5 Inhibition of SMYD3 promotes apoptosis in hepatoma cells upon serum deprivation. **A**: FCM analysis of HepG2 cells 48 h after transfection ($P < 0.01$). **B**: TUNEL assay of cell apoptosis with siRNA suppression of SMYD3 expression in HepG2 (brown staining cells) ($P < 0.01$).

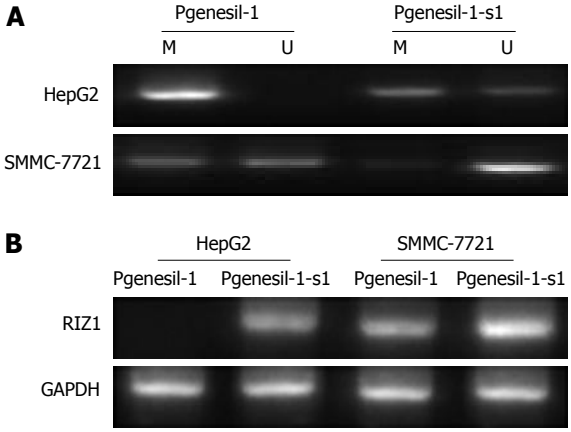

Figure 6 Inhibition of SMYD3 de-methylates RIZ1 promoter CpG islands and reexpresses RIZ1 in hepatoma Cells. **A**: Methylation specific PCR (MSP) assay of RIZ1 promoter. M: Methylated; U: Unmethylated; **B**: RIZ1 mRNA expression, GAPDH served as internal control (RT-PCR).

based functions, regulating fundamental processes such as gene transcription and DNA repair[16]. SMYD3, a gene located in 1q44 chromosome, encodes a protein with HMTase activity specifically on histone H3 at K4, and is involved in carcinogenesis and tumor progression[5]. In the present study, we confirmed that SMYD3 is overexpressed in HCC cell lines. To further investigate the function of SMYD3 in HCC carcinogenesis and tumor progression, RNAi technology was applied to specifically knock down SMYD3 expression in HCC cell lines. We demonstrated that depletion of SMYD3 reduced hepatoma cell growth, migration and induced cell apoptosis upon serum deprivation. Our findings suggest that SMYD3 plays pro-oncologic role in HCC development and progression. Interestingly, our results show that the expression level of SMYD3 in Hep3B cells differs significantly from that in HepG2 and SMMC-7721 cells. We hypothesized that the difference is possibly due to the HBV infectious state of the cells that we used; HBV positive in Hep3B, but negative in HepG2 and SMMC-7721 cells. However, this hypothesis needs to be further clarified.

SMYD3 is considered to function through its H3-K4 histone metylation, regulating expression of Nkx2.8[17,18], Wnt10B[19,20] and other genes involved in hepatoma cell-cycle regulation, cell proliferation and apoptosis. SMYD3 also upregulates genes linked to cell adhesion and invasion, such as ITGA5[21], COLQ[22], SELL[23], NEURL[24], and PECAM1[25], which may be responsible for cell migration regulatory role of SMYD3 in our experiment. To investigate whether SMYD3 might act on HCC oncological activity through TSG inactivation, we examined if RIZ1 activity could be regulated by SMYD3 in different hepatoma cells. RIZ1 was found to be downregulated in HepG2 cells with its promoter CpG hypermethylated, which was in line with enhanced SMYD3 expression, while knockdown of SMYD3 demethylated RIZ1 promoter and upregulated RIZ1 expression in these cells. In addition, different SMYD3 expression levels in hepatoma cells was also consistent with RIZ1 promoter methylation and mRNA expression in hepatoma cells in our results (data not shown) and in other results. HepG2 cells have been shown to lack RIZ1 expression due to promoter hypermethylation; in contrast, Hep3B cells do not show *RIZ1* promoter hypermethylation and express RIZ1 mRNA[26,27]. These results strongly suggest that SMYD3 plays a role in HCC development and progression, partly through RIZ1 promoter hypermethylation and RIZ1 inactivation.

As a histone/protein methyltransferase, SMYD3 mainly acts on histones or proteins; therefore, the mechanism by which SMYD3 regulate RIZ1 promoter CpG islands needs to be further clarified. SMYD3 includes a putative 428-amino acid protein containing a SET domain (codons 148-239) which is HMT, and a zf-MYND domain (codons 49-87), a typical zinc finger domain. The presence of a MYND-type zinc-finger domain in SMYD3 suggests that SMYD3 can recognize and bind particular sequences present in the promoter region of downstream genes through its MYND zinc finger. The specific SMYD3

binding elements (SBE) in target DNA are 5'-CCCTCC-3' or 5'-GGAGGG-3', which are present in the promoter regions of SMYD3 downstream genes, such as Nkx2.8[5]. It is interesting to note that one SBE, 5'-GGAGGG-3', is present in the promoter region of RIZ1[27]. A typical zinc finger domain in SMYD3 and SBE sequence in RIZ1 promoter strongly suggests that SMYD3 may act on RIZ1 promoter through its zf-MYND domain recognizing and binding to the SBE within the RIZ1 promoter. However, SMYD3 may also recognize SBE in other genes, such as DNA methyltransferases (DNMT), which are crucial for DNA methylation[28], and sequentially regulates RIZ1 promoter hypermethylation. There is other evidence suggesting that histone modification may interact with DNA methylation or may regulate DNA methylation[29]. In mammals, H3K9 methylation and CpG methylation shows a complex interplay in which each mark can influence the activity of the other.

Our results imply that, besides their gene-transacting role, SMYD3 and other H3-K4 methyltransferases might influence carcinogenesis and tumor progression by silencing TSGs through DNA methylation. In mammals, DNA methylation must be catalyzed by DNA methyltransferases, whether SMYD3 directly regulates DNMT expression, or SMYD3 changes the local conformation of RIZ1 promoter to facilitate DNMT congregation needs to be further investigated. Moreover, SMYD3 can di- and tri-methylate H3-K4, if its DNA methylation activity is dependent on H3-K4 transactivation, the H3-K4 hypermethylation patterns also needs to be established.

COMMENTS

Background

SMYD3, a H3K4 methyltransferase, was shown to be enhanced expressed in HCC, and involved in the growth of hepatocellular carcinoma (HCC) cells. Although transcriptional activation of downstream genes including Nkx2.8 and WNT10B gene was reported, the effect of SMYD3 on HCC development and the underlining mechanism remain unclear. RIZ1, one typical TSG with H3-K9 methyltransferase activity, was shown to lose its expression and tumor-suppressing activity in HCC, RIZ inactivation in HCC was mainly through its CpG promoter hypermethylation. In this article, whether SMYD3 regulates HCC proliferation, apoptosis and migration through RIZ1 promoter hypermethylation was initially investigated.

Research frontiers

In previous studies, histone metylation were thought to be irreversible. However, in recent studies, histone demethylase was found, which means that histone can be hypermethylated or demethylated in regulating gene expression. Since histone modification play important epigenetic regulating roles in gene transcription, this may provide new target for carcinoma therapy through hypermethylating or demethylating histone.

Innovations and breakthroughs

This article suggests H3-T4 histone methyltransferase regulates HCC biology, not only through oncogene transcription, but through interacting with H3-K9 HMT which mainly plays inhibitory role in oncogene expression, and through inhibiting TSG expression by promoter hypermethylation. These findings deepen our understanding of the interaction of different histone modification ways in gene transcription modification.

Applications

Clarifying the regulating models of H3-K4 methyltransferase, such as SMYD3, represents a potentiality for understanding oncogene expression and tumor suppressive gene under-expression in carcinogenesis and progress. By deepened understanding the key role of SMYD3 in HCC development may also provide new target for carcinoma treatment.

Terminology

SMYD3 SET and MYND domain-containing protein 3; RIZ1 retinoblastoma protein-interacting zinc finger gene1; TSG tumor suppressive gene; HMT Histone methyltransferase; DNMT DNA methyltransferases.

Peer review

The manuscript describes studies showing that several HCC cell lines and one HCC tissue sample express SMYD3 and is well described.

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RAPID COMMUNICATION

CYP2E1 Rsa I polymorphism impacts on risk of colorectal cancer association with smoking and alcohol drinking

Chang-Ming Gao, Toshiro Takezaki, Jian-Zhong Wu, Min-Bin Chen, Yan-Ting Liu, Jian-Hua Ding, Haruhiko Sugimura, Jia Cao, Nobuyuki Hamajima, Kazuo Tajima

Chang-Ming Gao, Jian-Zhong Wu, Min-Bin Chen, Yan-Ting Liu, Jian-Hua Ding, Division of Epidemiology, Jiangsu Province Institute of Cancer Research, Nanjing 210009, Jiangsu Province, China
Toshiro Takezaki, Department of International Island and Community Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
Haruhiko Sugimura, First Department of Pathology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan
Jia Cao, Molecular Toxicology Laboratory, Third Military Medical Universit School of Public Healthy, Chongqing 400038, China
Nobuyuki Hamajima, Department of Preventive Medicine Biostatistics and Medical Decision Making, Nagoya University School of Medicine, Nagoya 466-8550, Japan
Kazuo Tajima, Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan
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Correspondence to: Kazuo Tajima, Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan. ktajima@aichicc.jp
Telephone: +81-52-7642970 Fax: +81-52-7635233
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Abstract

AIM: To investigate associations between the *Rsa* I polymorphism of *CYP2E1* and risk of colorectal cancer.

METHODS: A case-control study was conducted with 315 colorectal cancer cases (105 colon, 210 rectal) and 439 population-based controls in Jiangsu Province of China. Genomic DNA samples were assayed for restriction fragment length polymorphisms in *CYP2E1* by PCR amplification followed by digestion with *Rsa* I. Information on smoking and alcohol drinking was collected using a questionnaire. Odds ratios (ORs) were estimated with an unconditional logistic model.

RESULTS: The proportional distribution of the *CYP2E1 Rsa* I c1/c1, c1/c2 and c2/c2 genotypes were 61.4%, 35.6% and 3.0% in controls, 60.6%, 33.7% and 5.8% in colon cancer cases, and 58.4%, 34.0% and 7.7% in rectal cancer cases, respectively. A significant difference was noted between controls and rectal cancer cases (P = 0.029), the c2/c2 genotype being associated with elevated OR (adjusted age, sex and status of the smoking and alcohol drinking) for rectal cancer (1.64, 95% CI, 1.12-2.41, *vs* c1 allele carriers), but not for colon cancer. In interaction analysis between the *CYP2E1 Rsa* I genotype and smoking and drinking habits, we found a significant cooperative action between the c2/c2 genotype and alcohol drinking in the sex-, age-adjusted ORs for both colon (4.74, 95% CI, 1.10-20.40) and rectal (5.75, 95% CI, 1.65-20.05) cancers. Among non-smokers, the *CYP2E1 Rsa* I c2/c2 genotype was also associated with elevated ORs in the two sites (1.95, 95% CI, 0.99-3.86 and 2.30, 95% CI, 1.32-3.99).

CONCLUSION: The results of the present study suggest that the *CYP2E1* c2/c2 genotype increases susceptibility to rectal cancer and the gene-environmental interactions between the *CYP2E1* polymorphism and smoking or alcohol drinking exist for colorectal neoplasia in general.

Key words: *CYP 2E1*; Gene polymorphism; Smoking; Alcohol drinking; Colorectal cancer

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INTRODUCTION

CYP2E1, a member of the cytochrome P450 superfamily, is involved in the metabolic activation of many low-molecular-weight compounds such as N-nitrosamines, aniline, vinyl chloride, urethane and alcohol[1]. N-nitrosamines present in tobacco and diet are well-recognized carcinogens involved in tumor development at various sites. Functional *CYP2E1* gene polymorphisms might therefore impact on susceptibility to cancer development.

A substitution polymorphism (G1259C) detected using the restriction enzymes *Pst* I or *Rsa* I has been associated

with decreased *CYP2E1* activity/inducibility[2-5]. The *Dra* I polymorphism is also associated with altered activity of *CYP2E1*, although *Dra* I is located in intron 6 and is not thought to affect gene transcription[6]. Activity of *CYP2E1* is also modulated by various physiological determinants, such as obesity[7], fasting[7] and liver dysfunction[8] and can be induced by ethanol[9]. In contrast, dietary isothiocyanates[10] and garlic[11,12], as well as some drugs, such as disulfiram[13] and chlormethiazole[14], inhibit its activity. A number of environmental factors may thus modify the cancer risk through altered *CYP2E1* enzyme activity.

Previous studies have shown inconsistent findings on *CYP2E1* polymorphism associations with cancer risk. Some studies demonstrated the common genotype or alleles to confer greater risk of oral[15], pharyngeal[15], esophageal[16,17] liver[18] and lung[19,20] cancers. On the other hand, increased risk of oral[21], nasopharyngeal[22], liver[23] and colorectal[24,25] cancers was observed with the rare genotype or allele carriers in other studies. Furthermore, some case-control studies failed to find a significant association between *CYP2E1* polymorphisms and risk of neoplasia of the oral cavity and pharynx[26], esophagus[27], stomach[28,29], lung[30-33], bladder[34] and colorectum[35]. The reasons for these inconsistent results are not clear, but one problem is a lack of sufficient investigation of gene-environmental interactions, including links with dietary and smoking habits. We hypothesized that environmental factors may alter the enzyme activity of *CYP2E1* and therefore modify cancer susceptibility due to *CYP2E1* polymorphisms. One earlier study in our laboratory showed that gene-environment interactions between the *CYP2E1* polymorphism and smoking have the potential to alter susceptibility to gastric cancer[36].

To investigate possible relations between *CYP2E1 Rsa* I polymorphisms and environmental factors (smoking and alcohol drinking) on the risk of colorectal cancers, we conducted a population-based case-control study in Jiangsu province, China.

MATERIALS AND METHODS

Subjects

We recruited colorectal cancer cases using data of Cancer Registries in Huian and Jintan Cities of Jiangsu Province of China, and also recruited cases who visited Jiangsu Province Cancer Hospital from these cities from August 2000 to September 2002. All were histopathologically diagnosed as having a primary colorectal cancer. Physicians at the hospital asked eligible cases to participate in our study, and doctors or nurses interviewed the subjects and collected blood samples from a peripheral vein after obtaining informed consent. Population-based controls were selected from healthy residents in eight villages or towns of Huian and Jintan Cities. Doctors of the public health center randomly selected one or two controls for each case, after matching for ethnicity, sex and age within 2 years using the records of residents at the local governmental office, and then asked eligible residents for their participation. Interviews and blood collection were performed as for the cancer cases. A few patients and residents refused to participate in our study, but the response rates were 97% for cases and 93% for controls. The ethics committee of Jiangsu Province Institute of Cancer Research approved this study.

Environmental factors

The items of our questionnaire covered smoking and drinking habits. Smokers were divided into never- and ever-smokers (current and former). Drinkers also were divided into two groups ($\geq$ 2 times/mo and < 2 times/mo) according to drinking frequency.

DNA extraction and genotyping of the CYP2E1

Whole blood was collected into EDTA-coated tubes and centrifuged for 15 min, and the buffy coat layer was isolated. Genomic DNA was extracted from 200 μL of buffy coat using a Qiagen QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA). The method for genotyping of the *CYP2E1* has been previously described[30]. In brief, PCR was used to amplify the transcription regulation region of *CYP2E1* that includes the *Rsa* I enzyme recognition site[3]. The primers were 5'-CCAGTCGAGTCTACATTGTCA and 5'-TTCATTCT GTCTTCTAACTGG. The PCR product was subjected to *Rsa* I restriction enzyme digestion and samples were then analyzed by electrophoresis in 5% polyacrylamide gels. There were three genotypes of *CYP2E1* resulting from digestion with the restriction enzyme *Rsa* I : the common homozygote c1/c1; the heterozygote c1/c2; and the rare homozygote c2/c2. Among 754 examined samples, PCR products could not be visualized for 2 cases and 6 controls.

Statistical analysis

Associations between the *Rsa* I polymorphism and colorectal cancer risk were estimated by OR, using the unconditional logistic regression model. We calculated adjusted ORs for age (continuous), sex, smoking and drinking habits. To investigate gene-environmental interactions, we also calculated (stratified analysis) ORs according to combinations of the *CYP2E1* genotypes and habits of the smoking and drinking, with *Rsa* I c1 allele carriers as the reference. The procedure LOGISTIC from the statistical package SAS was employed for the calculations. The probability of Hardy-Weinberg equilibrium was assessed by the χ^2 test.

RESULTS

Numbers of subjects were 190 male and 125 female with colorectal cancer, and 223 male and 216 female controls (Table 1). The proportion of females in controls was significant higher than in colorectal cases but the mean age did not differ between cases and controls. The proportional distributions of smokers and alcohol drinkers were significant higher in colorectal cancer cases than in controls.

The distributions of *CYP2E1 Rsa* I c1/c1, c1/c2 and c2/c2 genotypes were 61.4%, 35.6% and 3.0%, respectively, in controls, 59.1%, 33.9% and 7.0% in colorectal cases, 60.6%, 33.7% and 5.8% in colon cancer cases, and 58.4%, 34.0% and 7.7% in rectal cancer cases (Table 1). The proportional distribution significantly differed

Table 1 Background characteristics of colorectal cancer cases and their controls

	Controls *n* (%)	Colorectal cancer *n* (%)	Colon cancer *n* (%)	Rectal cancer *n* (%)
All of the subjects	439 (100.0)	315 (100.0)	105 (100.0)	210 (100.0)
Gender				
Males	223 (50.8)	190 (60.3)	65 (61.9)	125 (59.5)
Females	216 (49.2)	125 (39.7)	40 (38.1)	85 (40.5)
χ^2_{MH} (*P*)		6.70 (0.010)	4.19 (0.041)	4.34 (0.037)
Age (yr)				
< 40	42 (9.6)	44 (14.0)	14 (13.3)	30 (14.3)
40-49	75 (17.1)	54 (17.1)	15 (14.3)	39 (18.6)
50-59	150 (34.2)	88 (27.9)	30 (28.6)	58 (27.6)
60-69	131 (29.8)	85 (29.0)	26 (24.8)	59 (28.1)
> 70	41 (9.3)	44 (14.0)	20 (19.1)	24 (11.4)
χ^2_{MH} (*P*)		9.37 (0.053)	10.23 (0.037)	5.69 (0.224)
Mean age ± SD	55.7 ± 11.0	55.3 ± 12.7	56.4 ± 13.4	54.7 ± 12.3
P (*t* test)		0.6172	0.6325	0.3159
Smoking status				
Nonsmoker	284 (64.7)	176 (55.9)	61 (58.1)	115 (54.8)
Courrent and former	155 (35.3)	139 (44.1)	44 (41.9)	95 (45.2)
χ^2_{MH} (*P*)		5.99 (0.014)	1.59 (0.208)	5.91 (0.015)
Alcohol status				
Nondrinker	327 (74.5)	176 (55.9)	65 (61.9)	120 (57.1)
Current and former	112 (25.5)	139 (44.1)	50 (38.1)	90 (42.9)
χ^2_{MH} (*P*)		28.58 (0.000)	14.19 (0.000)	19.90 (0.000)
CYP 2E1 genotypes[1]				
c1/c1	266 (61.4)	185 (59.1)	63 (60.6)	122 (58.4)
c1/c2	154 (35.6)	106 (33.9)	35 (33.7)	71 (34.0)
c2/c2	13 (3.0)	22 (7.0)	6 (5.8)	16 (7.7)
χ^2_{MH} (*P*)		6.58 (0.037)	1.91 (0.385)	7.07 (0.029)

[1]Six controls and two cases were excluded because of unknown CYP2E1 genotype.

Table 2 CYP2E1 genotypes and risk of colorectal cancer

	Genotype	Cases (*n*)	Controls (*n*)	OR[1] (95% CI)	OR[2] (95% CI)
Colorectal cancer					
	c1/c1	185	266	1.00	1.00
	c1/c2	106	154	0.99 (0.72-1.35)	0.99 (0.72-1.35)
	c2/c2	22	13	1.54 (1.07-2.19)	1.55 (1.08-2.22)
Colon Cancer					
	c1/c1	63	266	1.00	1.00
	c1/c2	35	154	0.96 (0.61-1.52)	0.95 (0.60-1.51)
	c2/c2	6	13	1.36 (0.82-2.25)	1.40 (0.84-2.33)
Rectal cancer					
	c1/c1	122	266	1.00	1.00
	c1/c2	71	154	1.01 (0.71-1.44)	1.01 (0.71-1.44)
	c2/c2	16	13	1.61 (1.09-2.36)	1.64 (1.12-2.41)

[1]ORs were adjusted for age and sex in a logistic regression model. [2]ORs were adjusted for age, sex and status of smoking and alcohol drinking.

between control and colorectal (χ^2_{MH} = 6.58, P = 0.037) or rectal (χ^2_{MH} = 7.07, P = 0.029) cancer cases. The allelic distribution of the *Rsa* I polymorphism for controls was in Hardy-Weinberg equilibrium (χ^2 = 2.77, P > 0.05). It shows that the controls from general population are representative. The *CYP2E1 Rsa* I c2/c2 genotype was associated with significantly increased ORs for colorectal cancer (sex-, age- and habits of smoking and alcohol drinking adjusted OR = 1.55, 95% CI, 1.08-2.22) and rectal cancer (adjusted OR = 1.64, 95% CI, 1.12-2.41) (Table 2).

Table 3 shows the results of the multivariable analysis of smoking, alcohol drinking and *CYP2E1 Rsa* I c2/c2 genotypes and risk of colorectal cancer. The smoking habit was not associated with any increased OR for colon or rectal cancer, but alcohol drinking was linked with elevated ORs for colon (1.68, 95% CI, 0.97-2.89) and rectal (2.08,

Table 3 Logistic regression analysis on smoking, alcohol drinking and CYP2E1 c2/c2 genotypes and risk of colorectal cancer

	Colorectal cancer OR[1] (95% CI)	Colon cancer OR[1] (95% CI)	Rectal cancer OR[1] (95% CI)
Smoking	1.01 (0.69-1.47)	0.84 (0.49-1.44)	1.08 (0.70-1.67)
Alcohol drinking	1.91 (1.31-2.80)	1.68 (0.97-2.89)	2.08 (1.36-3.19)
CYP2E1 c2/c2	1.50 (1.05-2.15)	1.34 (0.81-2.23)	1.58 (1.08-2.32)

[1]Logistic regression model included age (continuous), sex, smoking (nonsmoker, current + former smoker), alcohol drinking (nondrinker, current + former drinker) and *CYP2E1* genotype (c1/c1 + c1/c2, c2/c2).

Table 4 Interaction between the *CYP2E1* genotype and the status of smoking and alcohol drinking, and the odds ratios (ORs) for colorectal cancer

	CYP2E1 genotype	Controls	Colorectal cancer		Colon cancer		Rectal cancer	
		n	*n*	OR[1] (95% CI)	*n*	OR[1] (95% CI)	*n*	OR[1] (95% CI)
Smoker								
No	c1/c1 + c1/c2	275	162	1.00	57	1.00	105	1.00
No	c2/c2	5	14	2.20 (1.31-3.70)	4	1.95 (0.99-3.86)	10	2.30 (1.32-3.99)
Yes	c1/c1 + c1/c2	145	129	1.34 (0.93-1.92)	41	1.11 (0.66-1.88)	88	1.48 (0.98-2.25)
Yes	c2/c2	8	8	1.41 (0.50-3.96)	2	0.91 (0.18-4.57)	6	1.75 (0.57-5.42)
Drinker								
No	c1/c1 + c1/c2	313	174	1.00	62	1.00	112	1.00
No	c2/c2	9	10	1.41 (0.89-2.24)	2	1.09 (0.50-2.38)	8	1.55 (0.95-2.53)
Yes	c1/c1 + c1/c2	107	117	1.86 (1.28-2.68)	36	1.50 (0.89-2.55)	81	2.07 (1.37-3.14)
Yes	c2/c2	4	12	5.42 (1.65-17.40)	4	4.74 (1.10-20.40)	8	5.75 (1.65-20.05)

[1]ORs were adjusted for age and sex.

95% CI, 1.36-3.19) cancers. The *CYP2E1 Rsa* I c2/c2 genotype significantly increased the OR for rectal cancer (1.58, 95% CI, 1.08-2.32).

Table 4 shows the results of interaction analysis of the *CYP2E1 Rsa* I polymorphism with smoking and alcohol drinking habits. Among nonsmokers, *Rsa* I c2/c2 was associated with elevated ORs for colon (1.95, 95% CI, 0.99-3.86) and rectal (2.30, 95% CI, 1.32-3.99) cancers. Among smokers with the *Rsa* I c2/c2 genotype, no increase in the OR for colon cancer was observed, and the slightly increased OR for rectal cancer also was not statistically significant.

Among carriers of the *Rsa* I c1 allele, alcohol drinking was significantly associated with an elevated OR for rectal cancer (2.07, 95% CI, 1.37-3.14). As compared with non-drinkers with the *Rsa* I c1 allele, drinkers with RsaI c2/c2 genotype had significant increased ORs for colon cancer (4.74, 95% CI, 1.10-20.40) and rectal cancer (5.75, 95% CI, 1.65-20.05).

DISCUSSION

The present study revealed a significant association between the *CYP2E1 Rsa* I c2/c2 genotype and risk of rectal cancer, as well as a notable interaction with smoking or alcohol drinking as environmental factors.

Previous investigations showed inconsistent findings. As regards colorectal cancer, Kiss *et al*[24] found the *CYP2E1* c2 allele to be significantly associated with colorectal cancer (OR: 1.91, 95% CI, 1.05-3.52) in a Hungarian population. Yu *et al*[25] found the *CYP2E1 Pst* I c2 allele to be a susceptibility factor for colorectal cancer, especially for colon cancer, and there is an apparent gene-environment interaction with salted food in a Chinese population. In a study from the Netherlands, although calculation of crude ORs revealed an increased risk for colorectal cancer associated with the variant *CYP2E1* genotype (OR: 2.2, 95% CI: 1.3-3.8), this was no longer evident after adjustment for age and gender[35].

The reason for the inconsistent findings for the *CYP2E1* polymorphism is unknown but clearly variation with ethnicity and gender could contribute to differences in influence on neoplasia. The rare *Rsa* I allele is considered to result in increased transcriptional activation of the *CYP2E1* gene[2,3], with elevated expression levels of *CYP2E1* mRNA and protein[3,37]. However, several studies demonstrated common genotype carriers to have the higher *CYP2E1* enzyme activity[4,5]. Differences in *CYP2E1* activity by ethnicity and gender have also been reported, females showing 25% lower activity than males[7,38]. Japanese appear to demonstrate 30%-40% lower activity of *CYP2E1* than Caucasians, even after taking account differences in body size[39].

In the present study, we found a gene-environmental interaction between the *CYP2E1* polymorphism and smoking. Thus, increased risk of colon or rectal cancer was associated with the *CYP2E1 Rsa* I c2/c2 genotype among smokers, but not non-smokers. A similar phenomenon was also found in another study[40]. It has been shown that among non-smokers, urinary styrene metabolites are significantly decreased in subjects with c1/c1 alleles of *CYP2E1* as compared with those with the c1/c2 genotype, whereas no significant differences in urinary metabolites were noted among smokers[41].

We found alcohol drinking to significantly increase risk of cancer development, especially in the rectum, and there was a significant interaction with the *CYP2E1 Rsa* I c2/c2 genotype in both the colon and rectum. Our results are consistent with previous investigations indicating that alcohol consumption is associated with an increased risk for cancers of many organs, such as oral cavity, pharynx, larynx, esophagus, breast, liver, ovary; colon, rectum, stomach and pancreas[42]. Chronic ethanol consumption may promote carcinogenesis by (1) production of acetaldehyde, which is a weak mutagen and carcinogen; (2) induction of *CYP2E1* and associated oxidative stress and conversion of pro-carcinogens to carcinogens; (3) depletion of S-adenosylmethionine and, consequently, induction of global DNA hypomethylation; (4) induction of increased production of inhibitory guanine nucleotide regulatory proteins and components of extracellular signal-regulated kinase-mitogen-activated protein kinase signaling; (5) accumulation of iron and associated oxidative stress; (6) inactivation of the tumor suppressor gene BRCA1 and increased estrogen responsiveness (primarily in breast); and (7) impairment of retinoic acid metabolism[43]. Alcohol also can affect the pharmacokinetics of drugs by altering gastric emptying

or liver metabolism by inducing *CYP2E1*[43]. *CYP2E1* is the key microsomal enzyme that metabolizes alcohol in the non-alcohol dehydrogenase pathway. Choi *et al*[44] also discovered that "ever"-drinking women with the *CYP2E1* c2 allele containing genotypes had an increased risk of developing breast cancer compared to non-drinkers with the *CYP2E1* c1/c1 genotype in the Korean population.

Finally, some limitations require discussion. Because the frequency of the *CYP2E1 Rsa* I c2/c2 genotype is lower in subjects, only relatively small numbers were available for subgroup analyses, with consequent reduction in the magnitude of statistical power and increase in the potential for random error. Another possible problem is selection bias for controls, these being recruited by local health staff, albeit from the general population with a high response rate. The proportional distribution of female in controls was higher than that in colorectal cases, which may have caused a lower prevalence of smokers and alcohol drinkers in the present controls, though we adjusted for sex and age in all statistical analyses.

In summary, the present study revealed a link between the *CYP2E1 Rsa* I polymorphism and increased risk of rectal cancer, with a significant interaction between the *Rsa* I polymorphism and smoking and alcohol drinking habits regarding development of both colon and rectal cancers. The data provide support for our hypothesis that cancer susceptibility with the *CYP2E1* polymorphisms may be altered by background environmental factors.

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COMMENTS

Background

Colorectal cancer is the fifth most commonly occurring cancer in China. Cytochrome P450 (CYP) enzymes in epithelial cells lining the alimentary tract play an important role in both the elimination and activation of (pro-) carcinogens. To estimate the role of *CYP2E1* in colorectal cancer development, we conducted a population based case-control study of colorectal cancer in Jiangsu Province of China.

Research frontiers

A lot of carcinogens from environment must be metabolized for their elimination and activation. Genetic polymorphisms of metabolizing enzymes may affect the metabolism of carcinogens and the risk of cancer formation in humans. Susceptibility to cancer is generally thought to be the sum of complex interactions between environmental and genetic factors. Thereby, how interaction between environmental and genetic factors is a hotspot of cancer epidemiological study. We Regarding to the hotspot, we studied interactions between *CYP2E1* and habits of smoking and alcohol drinking in colorectal cancer development.

Innovations and breakthroughs

In present study, we demonstrate a correlation between *CYP2E1 Rsa* I polymorphism in c2/c2 genotype is associated with rectal and not colon cancer. This increased risk associated with this polymorphism was negated in smokers. Furthermore, a significant cooperative action was seen between the c2/c2 genotype and alcohol consumption in both colon and rectal cancers.

Applications

This research exposes a screenable genetic risk factor and the effects of gene-environment interactions in identifying individuals at risk for colon or rectal cancer. These results have some theoretical and application values in the etiology and prevention of colorectal cancer.

Terminology

CYP2E1: cytochrome P450 2E1. *CYP2E1 Rsa* I polymorphism: *Rsa* I enzyme recognized polymorphism in *CYP2E1* gene.

Peer review

Gao *et al* demonstrate a correlation between *CYP2E1 Rsa* I polymorphisms in c2/c2 genotypes are associated with rectal and not colon cancer. This increased risk associated with this polymorphism was negated in smokers. Furthermore, a significant cooperative action was seen between the c2/c2 genotype and alcohol consumption in both colon and rectal cancers. The experiments contain appropriate controls and weightings of the data taking into consideration age, sex, smoking status and alcohol consumption. This research exposes a screenable genetic risk factor and the effects of gene-environment interactions in identifying patients at risk for colon or rectal cancer.

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RAPID COMMUNICATION

Mycobacterium avium subspecies *paratuberculosis* infects and multiplies in enteric glial cells

Leonardo A Sechi, Anne Ruehl, Niyaz Ahmed, Donatella Usai, Daniela Paccagnini, Giovanna E Felis, Stefania Zanetti

Leonardo A Sechi, Donatella Usai, Daniela Paccagnini, Giovanna E Felis, Stefania Zanetti, Department of Biomedical Sciences, Microbiology Section, University of Sassari, Viale S Pietro 43/B, Sassari 07100, Italy
Anne Ruehl, Department of Human Biology, Technical University of Munich, Hochfeldweg 2, D-85350 Freising-Weihenstephan, Germany
Leonardo A Sechi, Niyaz Ahmed, Stefania Zanetti, International Society of Genetic and Evolutionary Microbiology, Working Group on Mycobacterial Disease, Sassari, Italy
Niyaz Ahmed, Pathogen Evolution Group, Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India
Correspondence to: Leonardo A Sechi, Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica, Università degli studi di Sassari, Viale S. Pietro 43/B, Sassari 07100, Italy. sechila@uniss.it
Telephone: +39-79-228303 Fax: +39-79-212345
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Abstract

AIM: To establish the role of enteric glial cells during infection with *Mycobacterium avium* subspecies paratuberculosis (MAP) in Crohn's disease.

METHODS: In order to establish the role of enteric glial cells during infection with *M. avium* subspecies *paratuberculosis* (MAP) in Crohn's disease, Map adhesion experiments on enteric glial cells were performed as well as expression analysis of Map sigma factors during infection.

RESULTS: In this study, for the first time, we found a high affinity of MAP to enteric glial cells and we analyzed the expression of MAP sigma factors under different conditions of growth.

CONCLUSION: The fact that Map showed a high affinity to the glial cells raises concerns about the complicated etiology of the Crohn's disease. Elucidation of the mechanisms whereby inflammation alters enteric neural control of gut functions may lead to novel treatments for Crohn's disease.

Key words: *Mycobacterium avium* subspecies *Paratuberculosis*; Enteric glial cells; Inflammatory bowel diseases; Crohn's disease; Sigma factors expression

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INTRODUCTION

Several cell lines of the gut have been extensively studied such as intestinal epithelial cells, immune cells, smooth muscle cells and enteric neurons[1-4]. However, very little is known about enteric glial cells which belong to the enteric nervous system (ENS) along with the neurons[5]. The ENS, comprised of several plexuses, is located alongside the intestinal wall. The enteric glia are localised within the mucosal plexuses and the glial processes make close contact with the epithelial cell layers. The epithelial crypt bases, in particular, are surrounded by a dense network of glial cells[6]. These glial cells are small, star shaped with different processes of various length and shape[6]; they are morphologically and immunohistochemically different from microglia of the central nervous system and from all other peripheral glia and appear more closely related to astrocytes although functionally different[6]. Evidence for functional heterogeneicity of enteric glia has recently been described[7]. The enteric glial cells (EGC) could participate in neurotransmitter synthesis/inactivaction or in synaptic transmission. Moreover, they may also interact with intestinal capillaries to modulate endothelial permeability[8].

It has been shown that the enteric nervous system regulates intestinal barrier functions by regulating the zonula occludens-tight junctions[9]. Regarding EGC, it has been shown in a mouse model that EGC ablation results in fulminant intestinal inflammation due to disregulation and rupture of the epithelial intestinal barrier[8,10]. It has been suggested that there is a direct interaction between EGC (releasing soluble factors) and epithelial cells to enhance the intestinal epithelial cells[5]. Moreover they could have a role in the prevention of epithelial barrier disorganization and hyperproliferation during bacterial infection, inflammatory processes and neoplasia[11-13]. All these findings indicate that glial cells may be an important component of the intestinal mucosal defense system.

On the other hand, *Mycobacterium avium* subspecies *paratuberculosis* is the causative agent of Johne's disease, a chronic and incurable disease affecting ruminants and other animals[14]. Moreover, there is increasing evidence of its involvement in the enteric granulomatous syndrome of

Table 1 PCR primers, targets, position on the sequences

Target	Primer name	5'-3' sequence	Position[1]
16S	MAP16Sfor	ATCATGCCCCTTATGTCCAG	1179-1198
	MAP16Srev	TGAGACCGGCTTTAAAAGGA	1259-1278
sigA	sigAfor	GTACGCCACCCAGCTGATGTCG	714-735
	sigArev	CGTCGCGGCAGATCCACAT	787-805
sigB	sigBfor	GACCTGCTCGAGCACAGC	646-663
	sigBrev	CAGCACGCTGCGGATGTCGGTG	783-804
sigC	sigCfor	ACATCCGTCACCTGCAGTC	1088-1106
	sigCrev	GTCACCTCGACCAGATCCTC	1177-1195
sigD	sigDfor	CTTCCTGGCTTTCCTGTACG	249-268
	sigDrev	GATGGACTCGGTCGGGTAG	324-242
sigE	sigEfor	CACCCAGGAGACCTTCATCC	327-346
	sigErev	GACCATGTCCAGGAACAG	418-435
sigF	sigFfor	GGCAGCTCCTACAACACCTT	493-512
	sigFrev	ACTCCTGGTCCTCGATCCGG	597-616
sigF-like	sigF-likefor	ATGACCAACGCAATCGCTCC	1-20
	sigF-likerev	GGCATCCGGCGCAGTTCCA	88-107
sigG	sigGfor	GCGTTCGAAAGCTACGACAT	622-641
	sigGrev	CTGATACCACCCGGTGTACG	692-711
sigH	sigHfor	AATCTCAAGGCGTGGCTCTA	385-404
	sigHrev	TGATTTCCTCGGTCGGATAC	465-484
sigI	sigIfor	GGGCGACATCGACGACGTGC	150-169
	sigIrev	GACATCGCCCGGACGTTCGTG	235-255
sigJ	sigJfor	GCATCTACACGGCGGGCCTG	740-759
	sigJrev	GGCGAACCGGTGAACTTGT	857-875
sigL	sigLfor	CGTGATCGAACGGTCCTACT	399-418
	sigLrev	CCGCACCGCATAGTGTAGT	491-504
sigM	sigMfor	TGGCTGCACCGCATCGTG	238-255
	sigMrev	TCGGCGACCGGATAGTAGTCT	315-335
Other ECF-1	sigECF1for	GTTCTCCGCCGAGTCGATTT	310-328
	sigECF1rev	GTCGAATCCGAACACCTCAC	492-411
Other ECF-2	sigECF2for	GCATCCACACGATCGACAT	839-857
	sigECF2rev	GGTTGTGATGTTCCTGAACC	914-933
Other ECF-3	sigECF3for	GTCGGTCATGGGTTTCGT	780-797
	sigECF3rev	GCACCCAGCTCCAGTTTC	855-873
Other ECF-4	sigECF4for	GATCTCGTCGGCATCTCG	502-519
	sigECF4rev	TCCAATTCGTTTCGGAGATT	592-611
Other ECF-5	sigECF5for	GCAATTGACCCGTTCACC	945-962
	sigECF5rev	CTCCTCCAAAGCGGCTAAG	1020-1038
Other ECF-6	sigECF6for	TGCAAGGTAATTCGATCAAGG	419-439
	sigECF6rev	TCCCTCGTTGACCTGTGC	511-528

[1]longest sequence available (primary and TIGR annotation). ECF: Extracitoplasmatic function; TIGR: The Institute for Genomic Research.

humans called Crohn's disease[15-17]. For all these reasons we analyzed the interactions between EGC and M. paratuberculosis *in vitro*.

Sigma factors are part of the transcriptional regulators family and are responsible for binding to the RNA polymerase complex (composed of four distinct subunits); to recognize promoters and separate DNA strands[18]. In fact, every sigma factor has its own specificity, allowing the initiation of transcription of different subset of genes[18,19]. Some sigma factors (sigD, sigE, sigC, sigH and sigL) play a role in the virulence of *M. tuberculosis*[20,21]. The number and abundance of sigma factors reflect the ability of the bacterium to cope with various environmental conditions, stresses and insults[18,22].

In this study the expression of all 19 sigma factors of M. paratuberculosis were also tested during EGC infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions

M. paratuberculosis ATCC 43015, of human source was obtained from The RIVM, Bilthoven, The Netherlands. *M. paratuberculosis* for DNA extraction was grown in Mycobacteria Growth Indicator Tube (MGITl) medium supplemented with Mycobactin J and egg yolk. Murine enteric glial cells were previously isolated and characterized by Dr. Anne Ruhel[23].

Identification of M. avium subsp. paratuberculosis putative sigma factors

Identification of individual sigma factors in *M. avium* subspecies. paratuberculosis was obtained by text annotation searches and by BlastP similarity searches using *M. smegmatis* and *M. tuberculosis* H37Rv Sigma ORF as queries. Primers used in real time PCR are described in Table 1.

DNA extraction

DNA extraction was performed using the Ribolyser system (HYBAID, USA) and purified as previously described[17].

PCR conditions

Oligonucleotide sequences and position are reported in

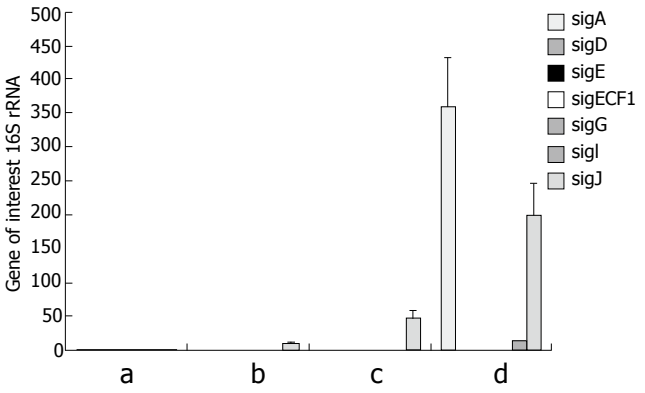

Figure 1 Relative quantification of expression of MAP sigma factors relative to the expression of the 16S rRNA MAP gene (calculated by the Biorad software) by Real Time PCR after different conditions of growth. a: After growth in 7H9 medium plus mycobactin J; b: Infection of EGC after 6 h of incubation; c: Infection of EGC after 48 h of incubation; d: Infection of EGC cells after 7 d of incubation.

Table 1. Specificity of primer pairs was ascertained with PCR with the following conditions: $MgCl_2$ 1.5 mmol/L, deossi Nucleotide Triphosphate (dNTPS) 150 μmol/L, primers 0.2 μmol/L each, Taq 0.025 U/μL reaction volume. After an initial denaturation of 3 min at 94℃, 30 cycles of 1 min at 94℃, 1 min at 58℃, 1 min at 72℃ and a final extension at 72℃ for 5 min were performed. Only the amplification products of the expected length were obtained.

RNA extraction

RNA was extracted using the Ribolyser instrument[17]. RNA was extracted from MAP cells after different conditions: (1) growth in 7H9 medium plus Mycobactin J, (2) after addition of lysozyme at 24 h and 7 d; (3) after infection of EGC line, after 6 h, 24 h, 48 h and 7 d. RNA was quantified for further experiments and samples were stored at -80℃ until further use. cDNA was prepared by an initial incubation at 65℃ for 10 min of the reaction mixture containing 100 μmol/L oligo-dT and 10 μL of extracted RNA; this first step was followed by a second incubation of 1 h at 42℃ of the mixture containing 50 U/reaction of reverse transcriptase (Mooloney-Murine Leukemia Virus M-MLV 200 U/μL), 0.1 mol/L buffer 5 × Tris HCl pH 8.3 (Life Technologies) with 150 mmol/L KCl, 7 mmol/L $MgCl_2$ and 20 mmol/L dithiothreitol (DTT); 0.1 mol/L DTT, 10 mmol/L dNTPs, 40 U/μL RNase inhibitor.

Real time PCR analyses

Real Time PCR was performed using the iCycler Detection System (Biorad) with the SyberGreen assay (Applied Biosystems). Gene expression of all the Sigma factors (except Extracytoplasmic function 3 ECF3) was determined using quantitative Real-Time PCR by comparing the fluorescence produced by test samples to that of the 16S rRNA gene expression (house-keeping gene). Five micro liters of cDNA was subjected to PCR (1 cycle at 94℃ for 1 min; 50 cycles at 94℃ for 30 s and at 60℃ for 40 s) with primers that amplified 16S rRNA, all 19 sigma factor genes. PCR was carried out with the following reaction-mixture in a total volume of 30 μL: 1 × of 10 × buffer, $MgCl_2$ 1.5 mmol/L, dNTPs 0.2 mmol/L, primers 0.5 μmol/L each, DNA polymerase 0.5 U/sample. PCR was performed in a 96 well plate, for each sample 2 wells were utilized to guarantee uniformity in results. The quantitative analysis of the data obtained was performed by Biorad's method of relative quantitation of gene expression (Bio-Rad Laboratories, USA).

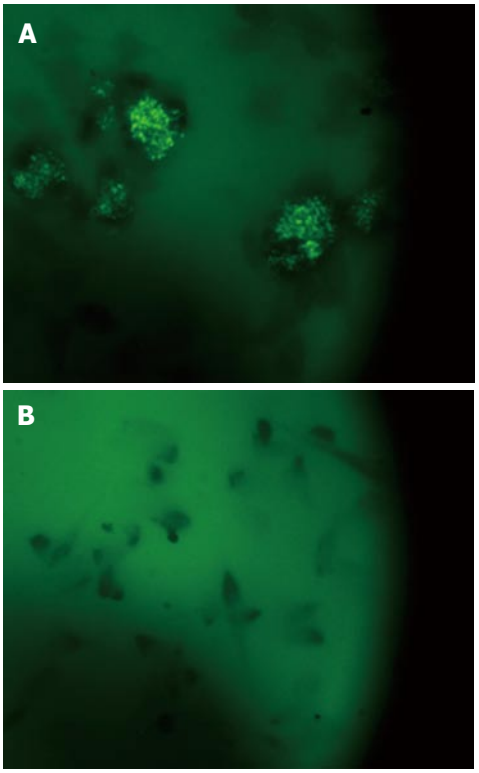

Figure 2 MAP cells stained with auramine rodhamine at 6 h after infection of EGC (**A**) and without infection (**B**) and visualized under a fluorescence microscope (× 100).

Infection experiments

Glial Enteric cell lines were cultivated in 24 well plastic dishes. The EGCs were cultivated in Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum and 4 mmol of L-glutamine per liter. All tissue culture reagents were obtained from Sigma (Sigma Chemical Co.). Cells were seeded at 2×10^4 cells per well and incubated at 37℃ in 5% CO_2. EGC cells were used when they were semiconfluent. MAP grown over night in 7H9 broth (with mycobactin J) was diluted in cell medium. One milliliter containing 10^6 bacteria was added to each well containing EGCs (cells were infected at a ratio of bacteria/cells of 10:1). After two hours infected EGCs were washed with cell medium containing Kanamycin 100 μg/mL and incubated with the same medium. Cells were stained with auramine rodhamine at the different incubation times (6 h, 48 h and 7 d at 37℃) to visualize MAP, and counted as previously described[17].

RESULTS

Expression of sigma factors in different conditions of Growth

7H9 broth plus mycobactin J: MAP during growth in

broth 7H9 supplemented with Mycobactine J, expressed several sigma factors as expected (Figure 1A). SigA, Sig D, SigE, Sig G and Sig I were among the most expressed ones as compared to the 16S rRNA expression taken as house keeping gene whereas ECF1 and sig J were expressed in lower quantity (Figure 1A).

Infection of EGC: The expression of the 19 sigma factors of MAP was observed and quantified after growth infection of EGC at 6 h, 48 h and 7 d (Figure 1 B-D). Soon after infection, expression of all sigma factors shut down, except for sig J which was expressed in high quantity (Figure 1B). The expression of sig J increased after 48 h (Figure 1C) and 7 d. SigA was overexpressed after seven days (Figure 1D), at the same time an expression of sig I was also detected. Figure 2 reveals an example of EGC infection by MAP where bacteria were visualized within the intestinal glial cells after staining with auramine rodamine as compared to control cells not infected.

DISCUSSION

Enteric glias are distinct from all other glial cell types[5,7]. No gastrointestinal disorder has been reported linked to glial defect yet, most probably because subtle changes in glial function might be involved in the etiopathogenesis of enteric disorders. Crohn's disease with neuroinflammation and neurodegeneration components may be associated with EGC alteration[23-25]. Indeed EGC interact with enteric neurons, endothelial cells, immune cells and the intestinal epithelium; all these factors can contribute to the pathogenesis of Crohn's disease. In our experiments, we found that MAP has a high affinity to EGCs. Experiments "*in vitro*" show a high adhesion and intracellular multiplication as confirmed by the active expression of sigA (the housekeeping sigma factor) after seven days of infection along with the expression of sigJ and sigI (expressed in different conditions of cellular stress).

The fact that this dangerous intestinal pathogen has a demonstrated affinity to the glial cells and that MAP has recently been reported in substantial percentages of Crohn's patients[15-17] raises concerns about the complicated etiology of the Crohn's disease.

MAP expression of sigma factors in EGC is very similar to sigma factor expression after infection of the Caco2 intestinal epithelial cell line (manuscript in preparation) and shows how there is a rapid change in gene expression after cell infection.

EGCs are an active part of an intestinal network system essential for a healthy and functional gut[23,26,27]. The role of EGC in Crohn's and other enteric diseases is not well studied and this is the first report that attempts to unravel interaction between an intestinal pathogen and EGC. Future work is certainly needed to elucidate this complex interaction.

COMMENTS

Background

Enteric glia might play a role in regulating barrier functions in mucosal epithelia. *Mycobacterium avium subspecies paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic and incurable disease affecting ruminants and other animals. Moreover, evidence of its involvement in Crohn's disease is accumulating. MAP enter the host through Peyer's patches and intestinal epithelium and are sampled by intestinal dendritic cells; they survives inside macrophages. Nothing is known about the role of enteric glial cells during MAP infection thus we performed this study to establish the role of enteric glial cells during infection with *Mycobacterium avium subspecies paratuberculosis* (MAP) in Crohn's disease.

Research frontiers

It is firmly established that intestinal inflammation is accompanied by functional and structural alterations of the Enteric Nervous System to which belong Enteric Glial Cells. In Crohn's disease patients, a compromised glial network that responds poorly to inflammatory stimuli has been suggested. Our report will open a new frontier on the interaction of MAP and enteric glia cells supporting the idea that Crohn's disease is associated with the invasion and persistence of a multi host pathogen as it is MAP.

Innovations and breakthroughs

This is the first research that highlights the interaction of a true enteric pathogen such as Map with Enteric glial cells. This study will open the research field in this matter.

Applications

Enteric glial cells may be the target of a series of cytokine modulators or drugs. Map will be recognized as a pathogen associated with intestinal disorders.

Terminology

EGCs: enteric glial cells; Map: Mycobacterium avium subs. Paratuberculosis; ENS: Enteric nervous system; Johne's disease: Enteric granulomatous infection of ruminants caused by Map; Crohn's disease: Enteric granulomatous chronic inflammation in humans with ulcerative colitis (UC) forms inflammatory bowel disease (IBD).

Peer review

The manuscript by Sechi *et al* describes the expression of MAP sigma factors during infection of enteric glial cells. The first findings that sigma factors are differentially expressed during infection are very promising.

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RAPID COMMUNICATION

Early prediction of major depression in chronic hepatitis C patients during peg-interferon α-2b treatment by assessment of vegetative-depressive symptoms after four weeks

Geert Robaeys, Jozef De Bie, Marieke C Wichers, Liesbeth Bruckers, Frederik Nevens, Peter Michielsen, Marc Van Ranst, Frank Buntinx

Geert Robaeys, Department of Gastroenterology and Hepatology, Ziekenhuis Oost Limburg, Genk, Belgium
Jozef De Bie, Department of Psychiatry, Ziekenhuis Oost Limburg, Genk, Belgium
Marieke C Wichers, Department of Psychiatry and Neuropsychiatry, South Limburg Mental Health Research and Teaching Network, EURON, Maastricht University, Maastricht, The Netherlands
Liesbeth Bruckers, Center for Statistics, Limburgs Universitair Centrum, Diepenbeek, Belgium
Frederik Nevens, Department of Hepatology, Katholieke Universiteit Leuven, Leuven, Belgium
Peter Michielsen, Division of Gastroenterology and Hepatology, University Hospital, Antwerp, Belgium
Marc Van Ranst, Laboratory of Clinical and Epidemiological Virology, Department of Microbiology and Immunology, Rega Institute and University Hospitals, Katholieke Universiteit Leuven, Leuven, Belgium
Frank Buntinx, Department of General Practice, Katholieke Universiteit Leuven, Leuven, Belgium and Department of General Practice, Maastricht University, The Netherlands
Supported by a received logistic support by Schering Plough
Correspondence to: Dr. Geert Robaeys, Ziekenhuis Oost Limburg, Department of Gastroenterology and Hepatology, Schiepse Bos 6, B 3600 Genk, Belgium. geert.robaeys@zol.be
Telephone: +32-89-326505 Fax: +32-89-327916
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Abstract

AIM: To study the predictive value of the vegetative-depressive symptoms of the Zung Depression Rating Scale for the occurrence of depression during treatment with peg-interferon α-2b of chronic hepatitis C (CHC) patients.

METHODS: The predictive value of vegetative-depressive symptoms at 4 wk of treatment for the occurrence of a subsequent diagnosis of major depressive disorder (MDD) was studied in CHC patients infected after substance use in a prospective, multicenter treatment trial in Belgium. The presence of vegetative-depressive symptoms was assessed using the Zung Scale before and 4 wk after the start of antiviral treatment.

RESULTS: Out of 49 eligible patients, 19 (39%) developed MDD. The area under the ROC curve of the vegetative Zung subscale was 0.73, $P = 0.004$. The sensitivity at a cut-point of > 15/35 was 95% (95% CI: 74-100). The positive predictive value equalled 44% (95% CI: 29-60).

CONCLUSION: In this group of Belgian CHC patients infected after substance use, antiviral treatment caused a considerable risk of depression. Seven vegetative-depressive symptoms of the Zung scale at wk 4 of treatment predicted 95% of all emerging depressions, at a price of 56% false positive test results.

Key words: Interferons; Hepatitis C; Chronic; Substance-related disorders; Depression; Zung self rating scale; Prognosis

Robaeys G, De Bie J, Wichers MC, Bruckers L, Nevens F, Michielsen P, Van Ranst M, Buntinx F. Early prediction of major depression in chronic hepatitis C patients during peg-interferon α-2b treatment by assessment of vegetative-depressive symptoms after four weeks. *World J Gastroenterol* 2007; 13(43): 5736-5740

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INTRODUCTION

Following the recent expert consensus, patients with chronic hepatitis C (CHC) should now receive interferon (IFN) and ribavirin regardless of psychiatric status, except in uncontrolled psychiatric disease[1-3]. As 23%-44% of patients treated with IFN develop depressive symptoms during treatment[4,5] and since, more patients are treated, interferon-induced psychiatric illness is likely to become an increasingly important clinical problem[4,6-14].

IFN-induced depression appears to be a depressive disorder that is unusually responsive to antidepressant treatment. Approximately 80% of patients are responsive within 4 wk[15]. This high response rate may be related to the usually short duration of this illness when treatment is initiated, to the mild to moderate severity of illness in most cases[1], but also to the mainly interferon-induced influences on the central nervous system[16].

Based on the results of a small scale study ($n = 16$), it has been hypothesised that vegetative symptoms are

early predictors for the emergence of full depression[9]. We wanted to confirm or refute this finding in a larger patient group of CHC positive substance users, classically considered as predisposed for depression[17].

Therefore, the predictive value of an increase in the score of the seven vegetative-depressive symptoms from the 20-item Zung Depression Rating Scale[18] was studied in a new study in substance users, using the emergence of a DSM-IV based major depression during treatment as the outcome measure.

MATERIALS AND METHODS

Subjects

Previously untreated patients, 18 years or older, with compensated chronic HCV infection acquired after substance use, with a serum alanine aminotransferase level above the upper limit of normal, and positive serum HCV RNA were eligible for the study.

HCV patients infected after substance use were defined as patients who were HCV-positive and who had used cocaine, heroin or LSD at least once. All of them were currently or previously cared for in multidisciplinary and specialised programs, according to criteria defined in the Belgian guidelines for the treatment of CHC patients infected after substance use (Table 1)[1]. Patients, who had co-infections such as hepatitis B virus or human immunodeficiency virus, or patients with a diagnosis of uncontrolled neurological, cardiovascular, endocrine, haematological, hepatic or renal disease or patients with insufficient knowledge of the Dutch or French language were excluded.

The study was approved by the ethical review board of the Catholic University of Leuven Medical School and by the Medical Ethical Committees of each of the different centres, and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject prior to participation.

Methods

Patients were treated in a prospective, randomised, multi-center study in Belgium between November 2001 and February 2004 with pegylated interferon α-2b, 1.5 μg/kg weekly by subcutaneous injection in combination with ribavirin (administered orally, 1000-1200 mg/d, depending on body weight) or interferon α-2b PEN 3 MIU subcutaneously 3 times/wk in combination with ribavirin (administered orally, 1000-1200 mg/d, depending on body weight). The total duration of therapy for the two study medication arms was 48 wk if the patient was infected with genotype non-2-3 and 24 wk if the patient was infected with genotype 2-3. The patients were required to use effective birth control. A subgroup out of the study centers that collaborated to the study, also recorded the emergence of depression. In this group we systematically investigated the occurrence of depression during therapy in patients who were not already depressed at baseline.

A complete medical history, physical examination, laboratory blood test and liver biopsy were performed before study entry. A liver biopsy was performed before randomisation.

Table 1 Characteristics at baseline of chronic hepatitis C patients infected by substance use in a Belgian population eligible for treatment in a randomised trial

Characteristics at baseline	*n*	Thrice in wk 3 MIU interferon	Weekly pegylated interferon	Total	Wilcoxon rank sum test or fishers exact test
Age at baseline (yr)	49	37	37	37	0.99
at exposure (yr)	49	27	28	27	0.55
at infection (yr)	49	33	36	35	0.58
Body mass index	33	25	25	25	0.93
Genotype (%)					
-non 2-3	25	73	36	53	0.02[a]
-5	22	27	64	47	
Gender (%)					
F	11	26	19	22	0.73
M	38	74	81	78	
Methadone user (%)	49	39	35	37	0.77
Drug user active (%)	48	59	54	56	0.77
IVDU (%)	49	91	92	92	1.00
Activity[1]					
A0 + 1	40	81	96	89	0.17
A2 + 3	5	19	4	11	
Fibrosis[1]					
F0 + 1	20	38	50	44	0.19
F2 + 3	22	48	50	49	
-F4	3	14	0	7	
Steatosis[1]					
S0	20	40	52	47	0.73
S1	12	30	26	28	
S2 + 3	11	30	22	26	
ASAT at baseline (IU/L)	49	59	77	68	0.2098
ALAT at baseline (IU/L)	49	87	127	108	0.0635

IVDU: Intravenous drug users; ASAT: Aspartate aminotransferase; ALAT: Alanine aminotransferase. [1]According to metavir score for liver biopsy. [a]$P < 0.05$ between genotype-2-3 and non 2-3.

Risk determination

In total, 12 hepatologists in collaborating treatment centres (in whom 49 patients were treated) agreed to apply a 20-item Zung self-rating depression scale (SDS) at wk 0, 4 wk after start of treatment and after the treatment regimen was completed. Zung scale results could score between 25 (normal) and 100 points. A high score indicates a depressive attitude. A cut-off of equal to and higher than 60/100 was considered as indicative for diagnosable depression[18].

Cognitive-depressive and vegetative-depressive symptom dimensions were constructed as described earlier[19]. The vegetative-depressive symptoms (Diurnal variation, lack of sleep, loss of appetite, loss of sex drive, weight loss, constipation, fatigue) were scored from 1 to 4 (in the analysis multiplied with 5/4).

Outcome measures

Clinical diagnosis of depression was confirmed by a qualified psychiatrist on the basis of DSM-IV criteria for major depression[20].

Statistical analysis

Quantitative measures are expressed as mean or as median. Qualitative variables are presented as counts and percentages.

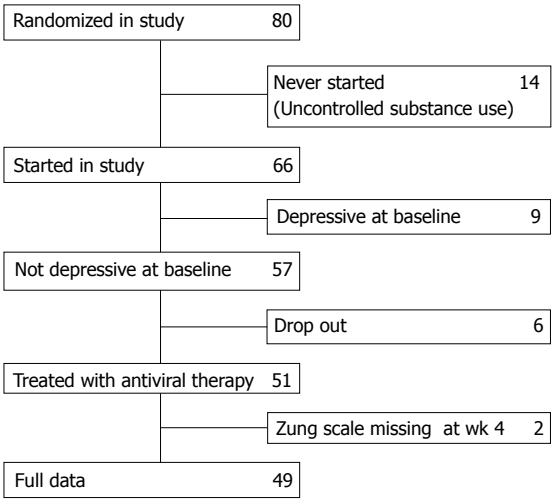

Figure 1 Flow chart of the patients included in the study.

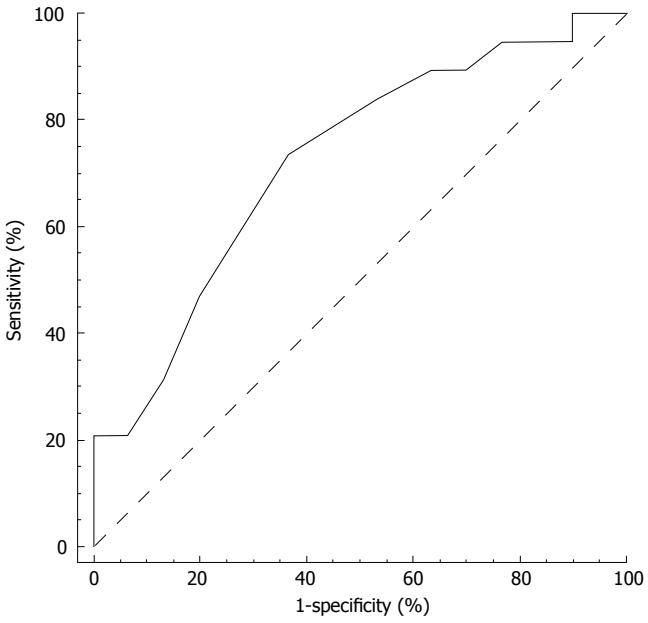

Figure 2 ROC prediction of depression based on vegetative Zung scale at wk 4.

The χ^2 test statistic and logistic regression methodology were used to compare two patient subgroups with respect to categorical outcome measures. *T*-tests for independent samples and linear regression methodology were applied to compare subgroups for differences in means of continuous variables. Non-parametric test statistics such as the Wilcoxon Rank Sum test were used if normality was absent.

In identifying an optimal cut-point for the Zung-vegetative symptoms score, we searched for a maximal sensitivity with a positive predictive value of at least around 50%. We report the full ROC curve, the AUC and its confidence interval for the four-week vegetative subscale of the Zung and the AUC and the *P*-values for possible alternative measures.

RESULTS

Participants

Eighty patients in whom chronic hepatitis C was diagnosed as acquired after substance use were randomised to treatment with either interferon α-2b or pegylated interferon α-2b combined with ribavirin and were followed by a hepatologist who accepted to apply the Zung rating scales. Fourteen patients never started the study because they were still consuming substances in an uncontrolled way. Nine had major depressive disorder (MDD) at baseline and were therefore excluded. For 8 patients data collection was incomplete. In total 49 eligible patients were included in this analysis (Figure 1).

Baseline characteristics

Demographic characteristics and clinical data for all eligible patients not having depression at baseline (n = 49) are summarized in Table 1. There were no baseline differences between the two treatment groups, with the exception of HCV genotype distribution (P = 0.02) and alanine aminotransaminase (ALT) level (P = 0.06).

Depression (DSM IV)

Nineteen patients became depressed during antiviral treatment. Median time to depression ranged from 4 to 26 wk and was distributed over this whole period with a mean of 10 wk.

Zung scale

At baseline there was no difference in total, vegetative or cognitive Zung score between patients who subsequently became depressed or not.

At wk 4 after treatment start the mean vegetative Zung scale was higher among the patients developing depression later on compared to other patients (P = 0.008). It decreased slightly from baseline to wk 4 (difference: -0.72; P = 0.49) for patients not getting depressed. Patients getting depressed increased on average 4.13 points on the vegetative Zung scale during the first four weeks of therapy (P = 0.03).

Diagnostic accuracy characteristics

The ROC curve for the prediction of depression emerging during treatment by the vegetative subscale at wk 4 is reported in Figure 2. The area under the curve (AUC) was 0.73 (95% CI 0.58-0.84; P = 0.004). At a cut-off point of > 17/35, the sensitivity and specificity of the vegetative subscale at wk 4 were 90% and 30%, respectively. The positive and negative predictive value equalled 45% and 82%. For a sensitivity of 95% (95% CI 74-100) the vegetative subscale criterion was > 15 and the corresponding specificity 23% (95% CI 10-42), the positive predictive value 44% (95% CI 39-60) and negative predictive value 88% (95% CI 47-100).

The increased vegetative Zung scale was not merely a measure of depression at that moment, but predicted the occurrence of depression scattered over the weeks and months afterwards. Results were not different according to gender, age and genotype.

The change in vegetative symptoms between wk 0 and 4 was marginally predictive (AUC = 0.68; P = 0.06).

The cognitive subscale at wk 4 was not able to predict a clinical diagnosis of depression (AUC = 0.62; $P = 0.15$). The total Zung at wk 4 did only have marginal predictive power (AUC = 0.67; $P = 0.05$).

DISCUSSION

The early presence of vegetative symptoms seems to be predictive for the occurrence of depression later on. In a small scale prospective study ($n = 16$)[9] it was found that the increase in the vegetative subscale score of the Montgomery Asberg Depression Rating Scale (MADRS) or the Hamilton Depression Rating Scale scales after one week of antiviral treatment following the start of antiviral treatment in CHC patients was predictive for MDD. We could confirm these findings in this prospective study during treatment with regular interferon or pegylated interferon in combination with ribavirin. Our study was larger and we had a more vulnerable population resulting in a higher number of patients becoming depressed during treatment. In this study, we found that a cut-off point of > 15/35 at the vegetative subscales at wk 4 resulted in a sensitivity of 95% at an acceptable positive predictive value of 44%.

This high sensitivity was found using a questionnaire of 7 items, part of the 20-item Zung scale. A similar sensitivity was found using the Minnesota Multiphasic Personality Inventory[21]. However, this rating scale includes 566 items and is more elaborate to perform in a routine outpatient clinic.

There was no relation between a higher score or a significant increase in the cognitive subscale at wk 4 and a higher risk of subsequent clinical depression. This is in agreement with the hypothesis formulated in reference[9].

Also, the Zung depression rating scale as a whole was not predictive for the development of depression in substance use patients, at least after exclusion of the patients that were already depressed at baseline. In other studies the baseline value was found to be predictive for patients with the Minnesota Multiphasic Personality Inventory[22], the Hamilton Depression Rating Scale score (HDRSS)[23] and the Centre for Epidemiological Studies-Depression Scale, CES-D[24] higher than a certain cut-off level[21,25,26]. However, in these studies people that were depressed at baseline were not always excluded[25,26].

In our study, an increased vegetative subscale score at 4 wk of treatment predicted diagnosis of major depression scattered over the weeks and months afterwards. This scatter is comparable to results found in other recent studies[4,7-10,25].

Although depressive symptomatology occurring in patients treated with interferon has usually been called 'depression' or 'major depressive episode', the more appropriate term according to new DSM-Ⅳ -TR should be 'substance induced mood disorder'. There are psychopathological (e.g., more irritability)[27], epidemiological (the gender difference in major depressive episode is different from the gender difference in interferon induced mood disorder)[28], genetic[29] and treatment (more patients than in major depressive episode respond well to SSRIs)[30] arguments to support this really to be a different disorder.

This study has some limitations. It was restricted to substance users with CHC infection and antiviral treatment. Therefore, it is not possible to extrapolate to other patient groups. However, from small studies there seems no difference in the development or the mechanism of depression in other patient groups[9,30]. Secondly, although larger than previous studies[9] our sample size is still relatively low resulting in large confidence intervals.

In conclusion, the development of depression can reliably be predicted at wk 4 after start of interferon treatment by an increase in vegetative symptoms of depression (subscale score) to at least 15/35. This results in a prediction with a sensitivity of 95%, at the price of 56% false-positive test results, which for a screening test is considered to be acceptable. Treatment with antidepressants may cure or prevent depression and so increase the treatment adherence. This may improve the SVR in patients evolving to depression and make the SVR comparable to SVR in non-depressed patients. To formally prove this, however, it should be the subject of another study.

COMMENTS

Background

Interferon derived treatment will remain the cornerstone for antiviral therapy in chronic hepatitis C patients in the near future. Interferon induced depression is a frequent side effect that may influence the outcome of the treatment. Since interferon-induced depression is unusually responsive to antidepressant treatment, it is important to detect the patients who are at risk for depression and who can benefit from antidepressive treatment.

Research frontiers

At this moment very few tools have been extensively studied to detect the patients at risk of depression during treatment with interferon. The Minnesota Multiphasic Personality Inventory is thoroughly studied but questioning 566 items makes it very elaborate to perform in a routine outpatient clinic.

Innovations and breakthroughs

Using only seven questions at week four of treatment from the vegetative-depressive symptoms of the Zung scale, it is possible to predict 95% of all emerging depressions, at a price of 56% false positive test results.

Applications

Treatment with antidepressants may cure or prevent depression and so increase the treatment adherence. This may improve the SVR in patients evolving to depression and make the SVR comparable to SVR in non-depressed patients. To formally prove the hypothesis that antidepressants may improve SVR in patients at risk for depression, should be the subject of further studies.

Peer review

Dr. Robaeys and co-workers-including the BASL Steering Committee and the Belgian Study Group: M Adler in Erasme University Hospital, Brussels; B Bastens in St Joseph Hospital, Liège; N Bourgeois, S Bourgeois in Ziekenhuis Netwerk Antwerpen (Stuivenberg), Antwerpen; R Brenard in St Joseph Hospital, Gilly; C Brixko in Hôpital de la Citadelle, Liège; Ph Caenepeel in Ziekenhuis Oost-Limburg, Genk; B Caucheteur in University Hospital St Pierre, Brussels; I Colle in University Hospital, Gent; C de Galocsy in Bracops Hospital, Brussels; J Delwaide in CHU Univertity of Liege, Liege; S Francque University Hospital Antwerpen, Antwerpen; J Henrion in Hôpital de Jolimont, Haine-Saint-Paul; J Holvoet in Ziekenhuis Netwerk Antwerpen (Middelheim), Antwerpen; Y Horsmans in Cliniques universitaires St-Luc UCL, Brussels; Ph Langlet in Clinique Edith Cavell, Brussels; P Laukens in St Jansziekenhuis, Brugge; V Lefebvre in CHR de Namur, Namur; H Louis1, P Michielsen10, JP Mulkay7, F Nevens in UZ Gasthuisberg KUL, Leuven; C Preux in Centre Hospitalier de Tivoli, La Louvière; G Robaeys6, H Van Vlierberghe8,

Ph Warzée in Reine Fabiola Hospital, Montignies sur Sambre - have presented a clinical study in which they aimed to show the predictive value of vegetative-depressive symptoms according to the Zung depression rating for subsequent clinical depression of HCV infected patients during antiviral treatment with interferon and Ribavirin after 4 wk of treatment. The manuscript is well written and presents a very interesting clinical study showing convincing data.

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RAPID COMMUNICATION

Acute pancreatitis in acute viral hepatitis

Pankaj Jain, Sandeep Nijhawan, Ramesh Roop Rai, Subhash Nepalia, Amit Mathur

Pankaj Jain, Sandeep Nijhawan, Ramesh Roop Rai, Subhash Nepalia, Amit Mathur, Department of Gastroenterology, SMS Medical College, Jaipur, Rajasthan, India
Correspondence to: Sandeep Nijhawan, Professor, Department of Gastroenterology, SMS Medical College, Jaipur, Rajasthan, India. dr_nijhawan@yahoo.com
Telephone: +91-141-2722335 Fax: +91-141-2560994
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Abstract

AIM: To elucidate the frequency and characteristics of pancreatic involvement in the course of acute (nonfulminant) viral hepatitis.

METHODS: We prospectively assessed the pancreatic involvement in patients with acute viral hepatitis who presented with severe abdominal pain.

RESULTS: We studied 124 patients with acute viral hepatitis, of whom 24 presented with severe abdominal pain. Seven patients (5.65%) were diagnosed to have acute pancreatitis. All were young males. Five patients had pancreatitis in the first week and two in the fourth week after the onset of jaundice. The pancreatitis was mild and all had uneventful recovery from both pancreatitis and hepatitis on conservative treatment. The etiology of pancreatitis was hepatitis E virus in 4, hepatitis A virus in 2, and hepatitis B virus in 1 patient. One patient had biliary sludge along with HEV infection. The abdominal pain of remaining seventeen patients was attributed to stretching of Glisson's capsule.

CONCLUSION: Acute pancreatitis occurs in 5.65% of patients with acute viral hepatitis, it is mild and recovers with conservative management.

Key words: Acute hepatitis; Pancreatitis; Viruses; Pain; Abdomen

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INTRODUCTION

Although hepatitis viruses have a strong tropism for hepatocytes, viral antigens have also been detected in other tissues such as the pancreas and gallbladder[1,2]. Several viral infections have been implicated as an aetiological factor of acute pancreatitis. The viruses most frequently responsible are mumps virus, Coxsackie B virus, Epstein-Barr virus and measles virus[3-6].

Acute pancreatitis is not uncommon in fulminant hepatic failure (FHF) and has been confirmed based on histology or serology[7-9]. Acute pancreatitis has been reported very rarely in acute (nonfulminant) viral hepatitis. It has been reported with hepatitis A (HAV), hepatitis B (HBV) and non-A-non-B (blood-borne) and hepatitis E virus (HEV) infection[10-20].

The aim of this study was to determine the frequency and characteristics of pancreatic involvement in the course of acute viral hepatitis.

MATERIALS AND METHODS

Materials

Patients seen at the gastroenterology clinic and ward of SMS hospital, Jaipur between January 2004 and December 2006 were included in this prospective study. Acute viral hepatitis was defined as patients with prodromal symptoms, deep jaundice, markedly raised transaminases, presence of markers of hepatitis B (positive for HBsAg, IgM anti-HBc, HBeAg, but negative for anti-HBe), hepatitis A (IgM anti-HAV), hepatitis C (anti-HCV), hepatitis E (IgM-anti-HEV) viruses in serum and ultrasound abdomen showing thick walled gallbladder and hypoechoic liver. Patients with other causes of acute hepatitis, chronic liver disease, history of alcohol intake and fulminant hepatic failure were excluded from the study.

The diagnosis of acute pancreatitis was based on pancreatic type abdominal pain, raised amylase and lipase levels to three times the upper limit of normal and ultrasound (US) or contrast enhanced computed tomography (CECT) of abdomen suggestive of acute pancreatitis.

Methods

The acute viral hepatitis group underwent hemogram, serum bilirubin, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase, prothrombin time, HBsAg, IgM anti-HBc, HBe Ag, anti-HBe, IgM anti-HAV, anti-HCV, IgM anti-HEV and ultrasound abdomen. Serum amylase and lipase, US of the abdomen and/or CECT were done in patients who had severe abdominal pain. Informed consent was obtained from each patient.

Table 1 Clinical and laboratory characteristics of 124 patients of acute viral hepatitis

	Acute viral hepatitis A (n = 16)	Acute viral hepatitis B (n = 54)	Acute viral hepatitis E (n = 54)
Male: Female	13:3	42:12	39:15
Age (yr)[1]	18.3 (8-35)	36 (20-74)	33.6 (17-62)
Clinical features			
Jaundice	15 (93.8)	54 (100)	54 (100)
Prodromal symptoms	16 (100)	54 (100)	54 (100)
Pain abdomen	4 (25)	8 (14.8)	12 (22.2)
Pruritis	4 (25)	9 (16.6)	19 (35.2)
Hepatomegaly (%)	6 (37.5)	16 (29.6)	21 (38.9)
Splenomegaly (%)	2 (12.5)	6 (11.1)	5 (9.2)
Hemoglobin (gm/dL)[1]	12.3 (9.6-14.5)	12.4 (9-15.1)	12.6 (9-14.7)
Platelets ($\times 10^5/mm^3$)[1]	2.5 (1.8-3.2)	1.8 (1.2-2.5)	1.6 (1-3.4)
Total leucocyte count ($\times 1000/mm^3$)[1]	7.9 (6.4-10.5)	8.4 (5-10.1)	7.2 (6-10.6)
Bilirubin (mg/dL)[1]	9.4 (0.67-35.8)	12.4 (3-36.5)	10.7 (3.3-26.3)
AST (IU/L)[1]	1969 (60-7715)	824 (223-3600)	1621.1 (240-3684)
ALT (IU/L)[1]	2134.3 (56-5165)	1024 (489-4900)	1830.6 (240-5549)
Serum alkaline phosphatase (IU/L)[1]	521.9 (196-1426)	424.5 (110-860)	324 (114-647)
Protein/Albumin (gm/dL)[1]	7.2 (6.5-7.8)/4.0 (3.5-4.6)	7 (6-8)/3.9 (3.4-4.8)	6.5 (5.5-8)/3.8 (3.4-4.6)
Prothrombin time prolongation[1]	4 (2-14)	5.5 (1-11)	6 (3-18)
Ultrasonography of the abdomen			
Thickened gallbladder	14 (87.5)	38 (70.4)	44 (81.5)
Hypoechoic liver	8 (50)	24 (44.6)	26 (48.1)
Gallbladder sludge	2 (12.5)	2 (3.7)	3 (5.5)
Ascites	1 (6.25)	3 (5.5)	26 (48.1)
Bulky pancreas	2 (12.5)	1 (1.8)	4 (7.4)

[1]Mean (range).

RESULTS

We had 124 patients with acute viral hepatitis over the three-year period, consisting of 94 males and 30 females, with a mean age of 32.7 years (range 8-65). The viral serology assays showed HBsAg and IgM-anti-HBc in 54 patients, IgM anti-HEV in 54 and IgM anti-HAV in 16 (Table 1). Twenty-four patients (19.4%) presented with history of severe abdominal pain, and 7 of these (29.2%) had evidence of acute pancreatitis. So, 5.65% of patients with acute viral hepatitis presented with acute pancreatitis. In the remaining 17 patients, the amylase and lipase were less than three times the upper limit of the normal. They did not have evidence of acute (calculous or acalculous) cholecystitis. We attributed the abdominal pain to stretching of the Glisson's capsule.

The clinical, laboratory and radiological profile of the patients with acute pancreatitis is given in Tables 2 and 3. The mean age was 23.9 (range 11-32) years, all were males. Abdominal pain occurred 2-30 (mean 12) days after the onset of jaundice. The duration of abdominal pain ranged from 24 to 120 h. None of the patients had a past history of jaundice, abdominal pain, alcoholism, trauma, hyperparathyroidism or drug intake. All patients had jaundice at presentation, mild hepatomegaly and epigastric tenderness. Three patients also had features of ileus.

On investigation, mean bilirubin was 16.4 mg/dL (range 5.8-32.4 mg/dL), mean AST 519 (range 182-1313) U/L and mean ALT 1371.1 (range 702-2438) U/L. Amylase and lipase ranged from 275-596 (mean 364.6) U/L and 520-7258 (mean 2495.4) U/L respectively. Serum lipid profile and calcium levels were normal in all patients. IgM anti-HEV, IgM anti-HAV, IgM anti-HBc were positive in 4, 2 and 1 patient respectively. US could detect pancreatitis in three patients, minimal ascites and biliary sludge in one patient. An abdominal CECT showed edematous and enlarged pancreas in all patients. There was no evidence of necrosis. The patient with sludge showed complete resolution in 2 wk. None of the other patients had evidence of biliary sludge during follow-up. The pancreatitis and hepatitis responded to conservative treatment in all patients. All patients are asymptomatic after a mean follow-up of 12 (range 8-24) mo.

DISCUSSION

Most cases of acute pancreatitis due to hepatitis viruses had been reported in association with acute liver failure (ALF). In the autopsy series of Ham and Fitzpatrick, 14 of the 42 (33%) patients with ALF had acute pancreatitis[8], the majority being of viral etiology. Similar findings had been reported by Parbhoo *et al*[9] in 21 out of 59 patients (36%) with ALF who had acute pancreatitis. Autopsy findings of pancreatitis in seven of 16 (44%) patients with fulminant viral hepatitis, versus two of 33 (6%) patients with fulminant hepatic failure secondary to halothane, support the role of viral infection rather than liver failure per se in causing pancreatitis[21].

There are only a few case reports of symptomatic pancreatitis occurring in the setting of acute viral hepatitis[10]. Most of the patients reported had presented with symptomatic pancreatitis in the early phase of the hepatitic illness[11-20] whereas Mishra *et al*[10] reported 6 patients at wk 2 or 3 after the onset of jaundice. Our series had five patients presenting in the first week and two in the fourth week after the onset of jaundice. One patient had biliary sludge and presented in the first week of jaundice. Ultrasound was done weekly in all the patients until clinical and biochemical resolution. Gallbladder wall thickness

Table 2 Clinical Profile of viral hepatitis patients with acute pancreatitis

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Age (yr)	11	30	32	21	30	20	23
Gender	M	M	M	M	M	M	M
Pain interval after onset of jaundice (d)	30	28	3	2	5	6	5
Pain duration (h)	96	72	120	96	24	48	72
Hepatomegaly (cm; subcostal)	5	2	2	3	3	2	3
Etiology	Hepatitis A	Hepatitis B	Hepatitis E	Hepatitis E	Hepatitis E	Hepatitis A	Hepatitis E
Recovery from pancreatitis (d)	8	6	12	6	3	6	4
Recovery from hepatitis (wk)	8	6	12	8	8	12	10

Table 3 Laboratory profile of viral hepatitis patients with acute pancreatitis

Parameter	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Bilirubin (mg/dL)	32.4	21.3	14.6	11.8	5.8	13.1	15.8
AST (IU /L)	682	1210	1313	182	388	1223	765
ALT (IU/L)	895	702	2238	702	412	2438	814
Alkaline phosphates (IU/L)	560	300	607	420	531	503	231
Albumin (mg/dL)	3.8	4	3.5	3.8	3.5	3.9	3.5
Prothrombin time prolongation (s)	14	11	12	9	11	10	18
Amylase (IU/L)	475	460	275	278	462	319	596
Lipase (IU/L)	7258	520	3297	990	760	2926	2060
Corrected Serum calcium (mg/dL)	9.2	9.1	9	8.9	9.2	9.3	8.1
Serum triglyceride (mg/dL)	140	168	131	165	141	160	154
Ultrasonography (Gallbladder)	Normal	Normal	Normal	Normal	Normal	Normal	Biliary sludge

(more than 3 mm) was found in 96.8% of patients, which is in agreement with a previous study by Sharma *et al*[22]. None of the two patients who presented later had any evidence of biliary sludge on US.

The etiology of pancreatitis was considered to be due to a hepatitis virus in all patients, as there was no evidence of gallstones, sludge, alcohol, drugs, trauma or metabolic causes. The remaining patient had biliary sludge on US. The disappearance of biliary sludge at 2 wk occurred a week later than previously reported[23]. None of the 7 patients with biliary sludge had an episode of acute pancreatitis and gallbladder contraction was similar in the hepatitis and control group in the Portincasa *et al*[23] group. So, the patient might had HEV-associated pancreatitis and had biliary sludge during the acute phase of the viral illness which resolved on follow-up ultrasound abdomen. Basaranoglu *et al*[24] reported a case of gallbladder sludge and acute pancreatitis induced by acute hepatitis A.

Acute pancreatitis in nonfulminant acute viral hepatitis covers the full range of clinical severity. Subclinical pancreatic involvement in viral hepatitis may occur more commonly than is appreciated[25]. The pancreatitis was mild as reported previously[10,13-20]. One of our patients had minimal ascites which resolved in 2 wk. There were no local or systemic complications and all had uneventful recovery from both pancreatitis and hepatitis. No relation was found between the level of amylase and severity of pancreatitis.

The frequency of acute pancreatitis in acute viral hepatitis in the present series is 5.65%, which is 2% higher than reported by Joshi *et al*[26] in their autopsy series of 108 cases. Joske *et al*[27] noted 8 cases of acute viral hepatitis out of 90 patients of acute pancreatitis.

The mechanism of pancreatitis in patients with acute viral hepatitis (nonfulminant) is unknown, and it may be multifactorial. One proposed pathogenesis of pancreatitis associated with hepatitis is the development of edema of the ampulla of Vater with obstruction to the outflow of pancreatic fluid[28]. There is no direct evidence for the routes by which hepatitis viruses reach the pancreas; however, the proposed routes are most likely blood or bile[14].

A more plausible mechanism for viral associated acute pancreatitis is direct inflammation and destruction of pancreatic acinar cell by the virus. This latter theory is supported by the autopsy finding of hepatitis B virus antigens within the cytoplasm of pancreatic acinar cells of patients with hepatitis B surface antigenemia[1,2]. It is possible that the severity of pancreatitis is related to the magnitude of exposure of pancreatic acinar cells to the hepatitis virus[2].

The hepatitis viruses might injure the pancreatic acinar cell membrane, resulting in the leakage of intracellular enzymes, and/or precipitate a network of intracellular events culminating in cell death by a mechanism analogous to hepatocyte necrosis[29]. Another mechanism can be the release and circulation of lysosomal enzymes from the inflamed liver with the activation of trypsinogen to trypsin.

When acute pancreatitis is associated with fulminant hepatitis, the virus may cause tissue damage directly, but there are several other factors which can play an important role in the development of pancreatitis (clinical or silent) and these include acute liver failure, hypotension, infections and drug induced damage[13]. Intrapancreatic

hemorrhage due to hypoprothrombinemia or disseminated intravascular coagulation may result in pancreatic damage and subsequent pancreatitis[8].

In conclusion, acute pancreatitis is not uncommon. In a patient with acute viral hepatitis and acute or disproportionate abdominal pain, acute pancreatitis should be kept as a possibility. Conservative treatment leads to recovery in all the patients.

COMMENTS

Background

The association of hepatitis viruses with acute pancreatitis in the setting of acute (nonfulminant) viral hepatitis is rare. The frequency and characteristics of pancreatic involvement in the course of acute viral hepatitis needs to be elucidated.

Research frontiers

Studies with larger number of patients of acute viral hepatitis with pain abdomen are necessary to determine the frequency and characteristics of pancreatitis as there are only case reports and one case series of six patients.

Innovations and breakthrough

Recent reports describe acute pancreatitis as a cause of acute or dispropionate abdominal pain complicating acute viral hepatitis.

Applications

Patients of acute viral hepatitis with severe pain abdomen should undergo serum amylase, lipase and ultrasonography or contrast-enhanced computed tomography of the abdomen to prove acute pancreatitis as a cause of abdominal pain. The prognosis of patients with acute pancreatitis in the setting of acute viral hepatitis is good and patients recover on conservative treatment.

Terminology

Acute viral hepatitis is defined as presence of prodromal symptoms, deep jaundice, markedly raised transaminases, presence of markers of hepatitis B (positive for HBsAg, IgM anti-HBc, HBeAg, but negative for anti-HBe), hepatitis A (IgM anti-HAV), hepatitis C (anti-HCV), hepatitis E (IgM-anti-HEV) viruses in serum and ultrasound abdomen showing thick walled gallbladder and hypoechoic liver . Patients with other causes of acute hepatitis, chronic liver disease, history of alcohol intake and fulminant hepatic failure were excluded from the study. The diagnosis of acute pancreatitis was based on pancreatic type abdominal pain, raised amylase and lipase levels to three times upper limit of the normal and ultrasound (US) or contrast enhanced computed tomography (CECT) of the abdomen suggestive of acute pancreatitis.

Peer review

This article describes the clinical findings of acute pancreatitis in acute viral hepatitis. The main content of the manuscript is good.

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RAPID COMMUNICATION

Triple non-invasive diagnostic test for exclusion of common bile ducts stones before laparoscopic cholecystectomy

Bahram Pourseidi, Amir Khorram-Manesh

Bahram Pourseidi, Department of Surgery, Kerman University of Medical Science, Kerman, Iran
Amir Khorram-Manesh, Department of Surgery Kungälv and Sahlgrenska University Hospitals, Göteborg, Sweden
Correspondence to: Amir Khorram-Manesh, MD, PhD, Department of General & Orthopedic Surgery, Kungälv Hospital, S-442 83 KUNGÄLV,
Sweden. amir.khorram-manesh@surgery.gu.se
Telephone: +46-303-98008 Fax: +46-303-98326
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Abstract

AIM: To evaluate the impact of a preoperative "triple non-invasive diagnostic test" for diagnosis and/or exclusion of common bile duct stones.

METHODS: All patients with symptomatic gallstone disease, operated on by laparoscopic cholecystectomy from March 2004 to March 2006 were studied retrospectively. Two hundred patients were included and reviewed by using a triple diagnostic test including: patient's medical history, routine liver function tests and routine ultrasonography. All patients were followed up 2-24 mo after surgery to evaluate the impact of triple diagnostic test.

RESULTS: Twenty-five patients were identified to have common bile duct stones. Lack of history of stones, negative laboratory tests and normal ultrasonography alone was proven to exclude common bile duct stones in some patients. However, a combination of these three components (triple diagnostic), was proven to be the most statistically significant test to exclude common bile duct stones in patients with gallstone disease.

CONCLUSION: Using a combination of routinely used diagnostic components as triple diagnostic modality would increase the diagnostic accuracy of common bile duct stones preoperatively. This triple non-invasive test is recommended for excluding common bile duct stones and to identify patients in need for other investigations.

Key words: Common bile duct stones; Laparoscopic cholecystectomy; Triple non-invasive diagnostic test

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INTRODUCTION

Surgery is the treatment of choice in symptomatic gallstone disease and is also recommended in asymptomatic patients due to complications followed by stone release in common bile duct[1-5]. Coexisting common bile duct stones (CBDS) occur in 7%-20% of all patients undergoing cholecystectomy[2,3,6]. Although intraoperative cholangiography was routinely performed to diagnose CBDS during pre-laparoscopic era, its use in the laparoscopic era has been debated[7-10]. Consequently, other techniques for diagnosing CBDS have been introduced[6-8].

Preoperative liver function test (LFT) results might be diagnostic for CBDS, if abnormal. However, some patients might have normal LFT despite coexisting CBDS[5,7]. Ultrasonography is the major diagnostic modality to diagnose gallstones, but is less helpful for diagnosing CBDS[1-7]. Computed tomography is rarely useful for diagnosing gallstones[5,8]. Magnetic-resonance-cholangio-pancreatography (MRCP) has high specificity and sensitivity and accuracy similar to that of Endoscopic-Retrograde-Cholangio-Pancreatography (ERCP), but its accuracy decreases if gallstones are small (< 4 mm) or if they are located near Vater's papilla[2,5,8,11]. In addition, MRCP is not widely available and unlike ERCP does not allow endoscopic extraction of stones[5,8,12]. ERCP is the most common technique used for both diagnostic and treatment of CBDS. It is however, expensive, invasive, technically demanding and associated with small but significant morbidity[6,7,13].

Clinical history of jaundice, pancreatitis or cholangitis, abnormal LFT results and or dilated common bile duct have been traditionally used to select possible candidates for ERCP preoperatively[5], but to the best of our knowledge, the accuracy of these parameters together in diagnosing and/or excluding CBDS has never been evaluated[5,6,8]. The aim of this retrospective study was to assess the accuracy of these variables alone and in combination for diagnosing/excluding CBDS in a consecutive group of patients with symptomatic gallstones disease operated on by laparoscopic cholecystectomy.

MATERIALS AND METHODS

From March 2004 to March 2006, all consecutive patients with ultrasound verified gallstones, in whom laparoscopic cholecystectomy was indicated, were studied retrospectively. The study was approved by the Afzali Hospital's ethical committee.

All information was collected based on a questionnaire including three different components. Patient's data and medical history, age at operation, gender, history of jaundice (obstructive *vs* non-obstructive), cholangitis or pancreatitis (acute *vs* non-acute) were recorded. The results of ultrasonography, preoperative liver functions tests (LFT), serum-total bilirubin (S-Bil), alkaline phosphatase (S-ALP) and white blood cells counts (WBC) were obtained. The following cut-off values were considered abnormal: S-Bil $\geqslant$ 1.5 dL/L, S-ALP $\geqslant$ 400 Ul/L and WBC $\geqslant$ 10 000/mm^3. CBDS diagnosis was established by either demonstrating a stone in CBD or a wide CBD (diameter $\geqslant$ 10 mm). All patients were operated laparoscopically. No intraoperative cholangiography was performed. All included patients (n = 200) were followed up 2-24 mo postoperatively, by means of a questionnaire, completed by telephone interviews or clinical visit if needed.

Statistical analysis

The statistical analysis was performed using SPSS (version 11.0). The association between occurrence of CBDS and different variables was analyzed using Chi-square test. Sensitivity, specificity, positive and negative predictive values were calculated for all variables individually and in combination. Student t-test with separate variance estimates was performed to test the demographic differences. Fisher exact test and χ^2-test were performed to compare between patients with a different number of suggested variables. $P < 0.05$ was considered statistically significant. Any patient with incomplete medical file or test results or any unclear information was excluded.

RESULTS

Two hundred patients, 43 men (21.5%) and 157 women (78.5%) were consecutively included in this study. The mean age of patients at the time of operation was 56.6 $\pm$ 18.2 and 51.6 $\pm$ 16.5 years for men and women, respectively. The average length of hospital stay was 30 (range 24-72) h for the whole group of patients, with no statistically significant difference between men and women.

Eighteen patients (9%) were found to have CBDS intraoperatively and additional 7 patients were found to have CBDS during the follow-up. The total number of patients with CBDS in this series was 25 patients (12.5%). The review of patient's clinical history did not reveal any clinical evidence for CBDS (jaundice, cholangitis or pancreatitis) in the majority of patients (89.5%). In the remaining patients (10.5%), besides cholangitis, all other clinical variables were more common in men. In 16 out of 25 patients with CBDS (8 women and 8 men), obstructive jaundice was the most frequent variable in both genders (Table 1). The association between clinical evidence for CBDS and occurrence of CBDS was calculated (Table 2). Thirteen patients had one positive clinical variable and 8 patients had two positive clinical variables. Higher number of positive clinical variables was not statistically significant for diagnosing CBDS. CBDS was more common in men with clinical evidence of the disease. The difference between men and women in this aspect was statistically significant (Chi-square: 6.56, df: 2, $P < 0.05$) (Table 2).

Table 1 Frequency of clinical variables in 200 patients with gallstones n (%)

Variables	Total	Women	Men
Obstructive jaundice			
Yes	16 (8)	8 (5.1)	8 (18.6)
No	183 (92)	148 (94.9)	35 (81.4)
Total	199 (100)	156 (100)	43 (100)
History of jaundice			
Yes	5 (2.5)	3 (1.9)	2 (4.7)
No	192 (97.5)	151 (98.1)	41 (95.3)
Total	197 (100)	154 (100)	43 (100)
Cholangitis			
Yes	5 (2.5)	5 (3.2)	0
No	192 (97.5)	149 (96.8)	43 (100)
Total	197 (100)	154 (100)	43 (100)
Acute Pancreatitis			
Yes	1 (0.5)	0	1 (2.3)
No	196 (99.5)	154 (100)	42 (97.7)
Total	197 (100)	154 (100)	43 (100)
History of Pancreatitis			
Yes	2(1)	1 (0.6)	1 (2.3)
No	195(99)	153 (99.4)	42 (97.7)
Total	197(100)	154 (100)	43 (100)

Table 2 Association between the number of positive clinical variables and CBDS n (%)

Patients	Negative history	1 positive findings	2 positive findings	Total
Men	34 (79.1)	6 (14)	3 (7)	43 (100)
Women	145 (92.4)	7 (4.5)	5 (3.2)	157 (100)
Total	179 (89.5)	13 (6.5)	8 (4)	200 (100)

The majority of patients in this cohort had a normal ultrasonography (n = 175, 87.3%). However, in 25 patients with CBDS (12 women and 13 men), 8 patients had a stone in common bile duct, 12 had obstruction in common bile duct, one had both a stone and obstruction of common bile duct, 3 had a widened common bile duct (> 10 mm), and finally one patient had widening of an intrahepatic bile branch. Positive ultrasonographic findings indicating CBDS were more common in men [13 out of 43 men (30%) and 12 out of 157 women (7%)] (Table 3). The difference in ultrasonographic diagnosis between men and women was statistically significant (Chi-square 20.23, df: 5 and $P < 0.05$).

The abnormal S-Bil and S-ALP results in men and women with CBDS are shown in Table 4. The majority of patients had normal laboratory results (> 85%). In 13 men (30.2%) serum bilirubin concentration was higher than 1.5 dL/L [compared to 11 women (7%)] and in 14 men (32.6%) the concentration of alkaline phosphatase was higher than 400 Ul/L [compared to 16 women (10%)]. All

Table 3 The results of ultrasound investigations in 200 patients with gallstones (per gender) *n* (%)

Patients	Men	Women	Total
CBDS	5 (11.9)	3 (1.9)	8 (4.1)
CBD obstruction	6 (14.3)	6 (3.9)	12 (6.1)
CBDS and obstruction	1 (2.4)	0 (0)	1 (0.5)
CBD diameter > 10 mm	1 (2.4)	2 (1.3)	3 (1.5)
Dilated intrahepatic bile duct branches	0 (0)	1 (0.6)	1 (0.5)
Normal	30 (69)	145 (92.3)	175 (87.3)
Total	43 (100)	157 (100)	200 (100)

CBD: Common bile duct; CBDS: Common bile duct stones.

Table 4 Results of liver tests in patients with gallstones (per gender) *n* (%)

Gender	S-bilirubin ⩾ 1.5 dL/L			Alkaline phosphatase ⩾ 400 UL/L		
	Yes	No	Total	Yes	No	Total
Men	13 (30.2)	30 (69.8)	43 (100)	14 (32.6)	29 (67.4)	43 (100)
Women	11 (7)	146 (93)	157 (100)	16 (10.2)	141 (89.8)	157 (100)
Total	24 (12)	176 (88)	200 (100)	30 (15)	170 (85)	200 (100)

patients with S-Bil ⩾ 1.5 dL/L had CBDS. These changes were more common in men and the difference between men and women was statistically significant (Chi-square 17.24, df: 1 and $P < 0.05$).

The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of each variable of medical history, LFT results and ultrasonography alone or in different combination for diagnosis/exclusion of CBDS was evaluated statistically (Table 5). As a single diagnostic test, ultrasonography had higher sensitivity (64%), specificity (97.1%), negative and positive predictive values (95% and 76.2%, respectively) than medical history, S-Bil and S-ALP. As a triple diagnostic modality, a combination of medical history, ultrasonographic findings and LFT results was proven to be the best diagnostic modality to exclude CBDS. White blood cells count had no impact in diagnosis of CBDS.

DISCUSSION

Although the majority of patients with gallstone disease have an uncomplicated surgical course, a few may become complicated due to occurrence of CBDS[1-3]. Due to fewer intraoperative cholangiographies during laparoscopic cholecystectomies[6,7], ERCP has been recommended for pre- or postoperative extraction of CBDS. It is however, an invasive investigation with high risk for complications and should be reserved for selected cases[12,14,15]. MRCP is not available in all institutions and its accuracy depends on the size and position of CBDS[4,11,12]. There is thus a need for easy-performing, non-invasive, and reliable test modalities to diagnose or exclude CBDS, by which selected patients can benefit from ERCP, MRCP or other expensive investigations[13-15]. Earlier studies on some clinical, laboratory or radiological variables have been performed[16,17]. There is however, as far as we know, no study performed evaluating a combination of these investigating modalities. The majority of patients with gallstones are routinely evaluated by their clinical history, ultrasonography and LFT (S-Bil and S-ALP)[3,5]. The main object of this study was to evaluate the efficacy of these non-invasive investigation methods for exclusion of CBDS diagnosis.

Table 5 Diagnostic value of each of the triple tests, alone and in combination *n* (%)

Variables	Sensitivity	Specificity	NPV	PPV
1. Medical history	44	92.5	90.5	38.1
2. Ultrasound	64	97.1	95	76.2
3. Alkaline phosphatase ⩾ 400 Ul/L	32	95	90	26.7
4. Serum bilirubin ⩾ 1.5 dL/L	30.3	76.2	89.2	25
2 + 3 + 4	80	82.9	96.7	40
2 + 3 or 2 + 4	32	97.1	90.9	61.5
2 + 1+ 3 or 4	84	81.7	97.3	39.6

NPV: Negative predictive value; PPV: Positive predictive value.

The importance of careful review of patient's history has been reported in many earlier studies. A focused positive patient's history may be an early indication of CBDS[3,6,16-18]. Hyperbilirubinemia preoperatively, had a high diagnostic significant in our study and is also reported by others[7,19]. Ultrasonographic investigation is a reliable diagnostic modality with high availability and lower cost. However, the result of the investigation depends highly on investigators experience[3,14,20]. In our study, ultrasonography was performed by one radiologist and our results are comparable with earlier reports[3,6]. As also earlier reported by others, our study indicates that in patients who lacked clinical, radiological and laboratory signs of CBDS, there is no need for performing ERCP[6,14,21-25]. It also indicates that by using patient's complete medical history (with special focus on the most common clinical complication of gallstone diseases such as pancreatitis), customary laboratory tests such as S-Bil and S-ALP and ultrasonography one may exclude CBDS diagnosis with high accuracy[26-29].

We used different variables as predictors *i.e.,* these variables were used to predict or explain the value(s) of one or more dependent variables (also referred to as dependent or outcome variables statistically). The positive predictive value (PPV), or precision rate, is defined as the proportion of patients with positive test results who are correctly diagnosed. Hence the PPV is used to indicate the probability that, in case of a positive test, the patient really has the specified disease. However there may be more than one cause for a disease and any single potential cause may not always result in the overt disease seen in a patient. In our study only ultrasonographic investigation has highest PPV followed by a combination of ultrasound investigation and LFT results. Since we were interested in evaluating the impact and usefulness of the negative ultrasonography or LFT results and the lack of clinical evidence (negative history of pancreatitis, cholecystitis and cholangitis) of CBDS, in our study, we used the negative predictive value (NPV), which is the proportion of patients with negative test results who are correctly

diagnosed. Higher NPV means then higher sensitivity for excluding CBDS as shown in our study; combining several tests in our study increased the negative predictive value and sensitivity of CBDS exclusion with almost equal specificity[16-18,30].

We concluded that patients with normal ultrasonography, LFT results and no clinical evidence of CBDS "negative triple test" (NPV of 97.3%) may undergo laparoscopic surgery without any need for preoperative MRCP or ERCP. The availability and non-invasiveness of this triple diagnostic test are additional benefits, which makes it more interesting.

COMMENTS

Background

Coexisting common bile duct stones (CBDS) may complicate the course of gallstone disease. During open surgical removal of gallstones (open cholecystectomy), cholangiography is performed to exclude, or if needed, to remove CBDS. With laparoscopic cholecystectomy becoming the first surgical choice for treatment of gallstones, preoperative cholangiography has not been performed routinely and the procedure itself has been debated. Consequently, other techniques have been used to exclude CBDS. These techniques, however, are either invasive, with consequent risk for complications, or only diagnostic; some of them not available at all hospitals. There is thus a need to establish a simple, non-invasive and cheap diagnostic method, available at all units, to identify patients with CBDS for further evaluation with invasive and more expensive techniques.

Research frontiers

The research front in this area is focused on developing different imaging techniques. The most promising technique is Magnetic-Resonance-Cholangio-Pancreatography (MRCP). It has high specificity and sensitivity and accuracy similar to that of ERCP (Endoscopic-Retrograde-Cholangio-Pancreatography), but its accuracy decreases if gallstones are small (< 4 mm) or if they are located near Vater's papilla. In addition MRCP is not widely available and unlike ERCP does not allow endoscopic extraction of stones. ERCP is the most common technique used for both diagnostic and treatment of CBDS. It is however, expensive, invasive, technically demanding and associated with small but significant morbidity.

Innovations and breakthroughs

By using already existed parameters; liver functions test, ultrasonography together with complete review of patient's medical history (triple diagnostic), we offer a simple, cheap and, for all clinicians, available triple technique, to diagnose or exclude CBDS without any extra cost or diagnostic delay.

Applications

The majority of patients without CBDS will be identified by this triple diagnostic technique, leaving the remaining few, to be investigated by MRCP or ERCP. The availability and non-invasiveness of this test are additional benefits, which makes it more interesting.

Terminology

Cholangiography: X-ray examination of the bile ducts following administration of a radiopaque contrast medium. Magnetic-Resonance-Cholangio-Pancreatography: is a medical imaging technique which uses magnetic resonance imaging to visualise the biliary and pancreatic ducts in a non-invasive manner. Endoscopic-Retrograde-Cholangio-Pancreatography: refers to the use of an endoscope; a thin, flexible tube with a tiny video camera and light on the end to diagnose and treat various problems of the GI tract (stomach, intestine, liver, pancreas, and gallbladder).

Peer review

Through retrospective study, the authors concluded that using a combination of routinely used diagnostic components as triple diagnostic modality would increase the diagnostic accuracy of common bile duct stones. The result is reasonable and persuasive.

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RAPID COMMUNICATION

Plasma and platelet serotonin levels in patients with liver cirrhosis

Đorđe M Ćulafić, Duško S Mirković, Miodrag D Vukčević, Jelena S Rudić

Đorđe M Ćulafić, Duško S Mirković, Miodrag D Vukčević, Jelena S Rudić, Clinic of Gastroenterology and Hepatology, Institute of Digestive Diseases; Institute of Medical Biochemistry; Institute of Pulmonary Diseases; Clinical Center of Serbia, Medical Faculty, Belgrade
Correspondence to: Đorđe M Ćulafić, Assistant Professor, Institute of Digestive Diseases, Clinical Center of Serbia, Koste Todorovica 6 street, Belgrade 11000, Serbia. dculafic@eunet.yu
Telephone: +387-11-2629811 Fax: + 387-11-2629811
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Abstract

AIM: To analyze the relationship between plasma and platelet serotonin levels and the degree of liver insufficiency.

METHODS: The prospective study included 30 patients with liver cirrhosis and 30 healthy controls. The degree of liver failure was assessed according to the Child-Pugh classification. Platelet and platelet poor plasma serotonin levels were determined.

RESULTS: The mean plasma serotonin level was higher in liver cirrhosis patients than in healthy subjects (215.0 ± 26.1 *vs* 63.1 ± 18.1 nmol/L; $P < 0.0001$). The mean platelet serotonin content was not significantly different in patients with liver cirrhosis compared with healthy individuals (4.8 ± 0.6; 4.2 ± 0.3 nmol/platelet; $P > 0.05$). Plasma serotonin levels were significantly higher in Child-Pugh grade A/B than in grade C patients (246.8 ± 35.0 *vs* 132.3 ± 30.7 nmol/L; $P < 0.05$). However, platelet serotonin content was not significantly different between Child-Pugh grade C and grade A/B (4.6 ± 0.7 *vs* 5.2 ± 0.8 nmol/platelet; $P > 0.05$).

CONCLUSION: Plasma serotonin levels are significantly higher in patients with cirrhosis than in the controls and represent the degree of liver insufficiency. In addition, platelet poor plasma serotonin estimation is a better marker for liver insufficiency than platelet serotonin content.

Key words: Serotonin; Plasma; Platelet; Liver cirrhosis

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INTRODUCTION

The serotonergic system plays a critical role in a wide variety of physiological and behavioral processes. In the circulation, serotonin synthesized by the intestinal enterochromaffin cells, is actively incorporated into platelets and stored in platelet dense-storage granules. Nearly all of the serotonin in circulating blood is concentrated in the platelets and minimally in plasma (which is the interactive pool). The integral membrane protein of mucosal epithelial cells is the major protagonist in regulating the extracellular serotonin concentration[1]. Serotonin is mostly metabolized into 5-hydroxyindoleacetic acid by monoamine oxidase in hepatic and lung endothelial cells[2]. The effects of serotonin are most prominent in the cardiovascular system, with additional effects in the respiratory system and the intestines. Vasoconstriction is a classic response to administration of serotonin. However, serotonin induces smooth muscle cell contraction and proliferation and stimulates endothelial cells to release vasodilating substances and acts as "helper agonist" of platelet aggregation in humans[3,4]. Altered concentrations of circulating serotonin have been implicated in several pathologic conditions including hypertension, primary pulmonary hypertension, liver cirrhosis, and psychiatric disorders[5-8].

The acute and chronic hepatic insufficiency gives rise to serotonin system changes, contributing to the development of hepatic encephalopathy, portal hypertension, and hyperdynamic circulation[9]. Hepatic encephalopathy is followed by changes of serotonin neurotransmission, including the catabolic enzymes, receptors, and metabolites[10]. After application of serotonin inhibitors (ketanserin and ritanserin), portal pressure is decreased in patients with liver cirrhosis, confirming the importance of serotonin in the pathogenesis of portal hypertension[11]. The aim of this study was to characterize the relationship between plasma and platelet serotonin levels and the degree of liver insufficiency.

MATERIALS AND METHODS

Subjects

In the period January-April, 2007, the prospective study included 30 patients with liver cirrhosis and 30 healthy controls, examined at the Institute of Digestive

Diseases, Clinical Center of Serbia, Belgrade. Hepatologic examinations were based on medical history, physical examination, laboratory tests, and liver biopsy. Laboratory tests included hepatocyte integrity, cholestasis, synthetic liver function, and specific (etiological) tests. Puncture liver biopsy was performed in 16 (53.3%) patients, using Menghini needle of 1.4 mm. The degree of liver failure was assessed according to the Child-Pugh classification.

Biochemistry

Platelet and platelet poor plasma (PPP) venous blood was collected in 3 mL original Vacutainer "BD" tubes with 75 g/L K_3EDTA 0.072 mL. Blood samples were taken between 8 and 9 am. Platelet number was immediately determined on "Coulter A^c T Diff" analyser. Platelet rich plasma (PRP) was obtained by low speed centrifugation (200 g, 10 min) on "Heraeus Digifuga GL". Platelet count was determined again and taken into consideration in final determination of serotonin concentration in platelets. Exactly 1 mL of PRP was centrifuged on 1000 × g for 10 min. The obtained PPP was separated, and together with platelet pellets, stored at -20℃, not longer then 20 d[12]. The whole number of platelet pellets and PPP serotonin samples were estimated in one series.

Platelet pellets were spiked with 10 μL N-methyl serotonin solution (Recipe, Munchen) as an internal standard. All pellets were diluted with 100 μL high performance liquid chromatography (HPLC) ultra pure water (Recipe, Munchen). Platelets were destroyed with 100 μL of 700 g/L perchloric acid (Merck Darmstad) and centrifuged at 10 000 × g for 5 min. 100 μL of PPP samples was deproteinized with 100 μL deproteinating reagent (Recipe, Munchen) and centrifuged in same way, as for platelets[13].

Twenty μL of the supernatants were analyzed by reverse phase HPLC (Recipe, Munchen) with original mobile phase for serotonin (Recipe, Munchen). Original, "Recipe" external standard solution has been used for calibration. The HPLC system consisted of "Bio-Rad AS 100" HRLC automatic sampling system with " Rheodine 7125 valve", "Bio-Rad 1350" HPLC pump, and "Bio-Rad 1640" electrochemical detection. Chromatographic data were calculated using the "Chrome Line V 4.20" HPLC software. Amperometric detection has been done on 0.6 V. Duration of chromatographic separation was 10 min.

Statistical analysis

Statistical analyses were performed using SPSS statistical software (SPSS for windows, release 10.0, SPSS, Chicago, Il). Descriptive statistics are presented as mean ± SE. Differences between groups were compared with parametric *t*-test because data had a Gaussian distribution. Because we performed 6 consecutive statistical analyses, we chose a level of significance 0.05/6 = 0.008 (α-adjustment according to the modified Bonferroni procedure).

RESULTS

The most common cause of liver cirrhosis was alcohol in 12 individuals (40.0%). The incidence of cirrhosis due to viral infection was lower with HCV in 6 (20.0%) and HBV in 5 subjects (16.6%); autoimmune diseases were quite rare with 3 cases (10.0%), while etiology was unknown in 4 (13.3%) cases. The patients were classified according to the Child-Pugh system. Child-Pugh class A included 12 (40%) patients, Child-Pugh class B 8 (26.6%), and Child-Pugh class C 10 (33.3%) patients. All patients had clinically evident portal hypertension, none of them had episodes of bacterial peritonitis, and none of them had a beta blocker medication.

The mean plasma free serotonin levels were much higher in liver cirrhosis patients than in healthy individuals. A statistical significant difference was found between serotonin plasma values in patients with liver cirrhosis and healthy subjects (215.0 ± 26.1 *vs* 63.1 ± 18.1 nmol/L; t = 3.868, $P < 0.0001$).

The mean platelet serotonin content was not significantly different in patients with liver cirrhosis compared to healthy individuals. There was no statistically significant difference between platelet serotonin content in patients with liver cirrhosis and healthy subjects (4.8 ± 0.6 *vs* 4.2 ± 0.3 nmol/platelets; t = 0.881, $P > 0.05$).

Plasma free serotonin levels were significantly higher in Child-Pugh grade A/B than in grade C patients (246.8 ± 35.0 *vs* 132.3 ± 30.7 nmol/L; t = 1.938, $P < 0.05$). However, platelet serotonin content was not significantly different between Child-Pugh grade C and grade A/B (4.6 ± 0.7 *vs* 5.2 ± 0.8 nmol/platelets; t = 0.48, $P > 0.05$).

In addition, plasma free serotonin levels were not significantly different between alcohol liver cirrhosis and cirrhosis of other etiology (143.9 ± 29.3 *vs* 224.84 ± 34.8 nmol/L; t = 1.6, $P > 0.05$). Also, platelet serotonin content was not significantly different between alcohol liver cirrhosis and cirrhosis of other etiology (4.2 ± 0.7 *vs* 5.3 ± 0.9 nmol/10^9 platelets; t = 0.91; $P > 0.05$).

DISCUSSION

Marasini *et al*[7] described a significant reduction of serotonin, determined by high-performance liquid chromatography, in platelets of 14 patients with liver cirrhosis, although levels of free circulating plasma serotonin were within the normal range. In the study of Beaudry *et al*[14], the whole-blood serotonin levels were significantly lower in 30 patients with cirrhosis than in age-matched controls, and no correlation was found between these levels and the severity of cirrhosis. This difference might be the result of low platelet count observed in patients with cirrhosis; however, in this series of patients, the significant difference persisted when the whole-blood serotonin levels were expressed by platelet count, but it was less expressed. Thus, in patients with cirrhosis, low whole-blood serotonin levels probably depend on reduction of both uptake, retention of serotonin by platelets, and low platelet number.

However, in the same study of Beaudry *et al*[14], unconjugated plasma serotonin levels, an indication of the active form of serotonin, were significantly higher in patients with cirrhosis than in the controls, and in patients with cirrhosis these levels were higher in Child-Pugh grade A than in grade C patients. In our study, we investigated levels of free or unconjugated serotonin. However, the levels of free serotonin in patients with liver cirrhosis were

also higher than in healthy subjects. Also, the levels of free serotonin in patients with Child-Pugh grade C is less than in grade A/B patients. In addition, platelet serotonin content was not significantly different when patients with liver cirrhosis were compared to healthy subjects.

The discrepancy of our and Beadry's study may be explained by the fact that whole blood is a similar, but not the same kind of biological material, as the platelet pellet is. In addition, we used HPLC as basic technique in our work, which significantly differs from Beadry's study, who also emphasized this difference in his study. The reasons for high levels of plasma serotonin in the liver cirrhosis could be slow uptake and storage of serotonin by the platelets (as could be the sequelae of the kinetic change of serotonin transport mechanisms) or abnormal serotonin release from dense granules of activated platelets.

Laffi *et al*[15] gave the evidence for significant reduction of substances that are deposited in thick (adenosine triphosphate and serotonin) and in alpha granules (B-thromboglobulin and platelet factor 4) in patients with liver cirrhosis in comparison to controls. It is supposed that platelet disorder in deposition of substances mentioned above, in patients with liver cirrhosis, is in relation to platelet activation, a condition defined as "platelet exhaustion".

The causes of platelet activation in liver cirrhosis are complex and not yet fully understood. Hyperdynamic portal circulation and retention in the spleen microcirculation in liver cirrhosis might stimulate platelets. Immunological and inflammatory phenomena in the liver tissue and its influence are other possible reasons for platelet activation. In addition, endotoxemia is often associated with severe liver cirrhosis and it causes platelet activation[16]. In liver cirrhosis, thrombocytopenia is associated with a shorter life span of platelets and is the result of constant platelet activation by cytokines (IL2, IL6, TNFα), it is mediated by subclinical DIC, and intensified elimination by reticuloendothelial system of the spleen and liver[17]. Platelet activation with the increase of both βTG serum concentration and elevation of platelet population (CD62P and CD63 as well as medial intensity of fluorescence CD62P and CD63) becomes higher as liver cirrhosis develops and thrombocytopenia rises. Concurrently with thrombocytopenia in liver cirrhosis, platelet CD63+ population increases, clearly indicating the platelet activation with elevated medial intensity of fluorescence CD62P and CD63[18].

Concentration of circulating serotonin in liver cirrhosis can be influenced by other factors, such as altered serotonin catabolism due to an elevated activity of monoamino oxidase and impaired metabolism of tryptophan, as serotonin precursor[19]. Impaired metabolic function in liver cirrhosis contributes to elevated plasma serotonin. Moreover, vasoactive substances, produced in the splanchnic circulation, bypass the liver in the presence of porto-systemic collaterals and directly enter the systemic circulation. In conclusion, plasma serotonin levels are significantly higher in patients with cirrhosis than in the controls, and represent the degree of liver insufficiency. In addition, PPP serotonin estimation is a better marker of liver insufficiency than platelet serotonin content.

COMMENTS

Background

The acute and chronic hepatic insufficiency gives rise to serotonin system changes, contributing to the development of hepatic encephalopathy, portal hypertension, and hyperdynamic circulation. In patients with liver cirrhosis, low whole-blood serotonin levels depend probably on reduced uptake, retention of serotonin by platelets, and low platelet number. It is supposed that reduced platelet deposition of substances in thick and alfa granules, is in relation to platelet activation, caused by hyperdynamic circulation and endotoxemia. Also, concentration of circulating serotonin in liver cirrhosis can be influenced by other factors, such as altered serotonin catabolism due to elevated activity of monoamino oxidase and impaired metabolism of tryptophan, as a precursor of serotonin.

Research frontiers

The serotonergic system plays a critical role in a wide variety of physiological and behavioral processes. Altered concentrations of circulating serotonin are implicated in several pathologic conditions including hypertension, primary pulmonary hypertension, liver cirrhosis, and psychiatric disorders. The highlight of our research was to characterize the relationship between plasma and platelet serotonin levels and the degree of liver insufficiency.

Innovations and breakthroughs

In Beadry's study, unconjugated plasma serotonin level, an active form of serotonin, was significantly higher in patients with cirrhosis than in the controls, and in cirrhotics this level was higher in Child A than in Child C patients. In our study, the levels of free serotonin in patients with liver cirrhosis were also higher than in healthy subjects. However, the levels of free serotonin in patients with Child C cirrhosis were less compared to Child A/B patients. Furthermore, platelet serotonin content was not significantly different in cirrhotics compared to healthy controls. The discrepancy of our and previous study could be explained by the fact that we used high performance liquid chromatography as basic technique in our work, which significantly differs from the technique used in the previous study.

Applications

The results in the study suggest that plasma serotonin levels may represent the degree of liver insufficiency. Also, platelet poor plasma serotonin estimation is a better marker for liver insufficiency than platelet serotonin content.

Terminology

Serotonin: Serotonin is a vasoactive substance, synthesized by the intestinal enterochromaffin cells, which is actively incorporated into platelets and stored in platelet dense-storage granules. Integral membrane protein of mucosal epithelial cells is the major protagonist in regulating the extracellular serotonin concentration. Serotonin is mostly metabolized into 5-hydroxyindoleacetic acid by monoamine oxidase in hepatic and lung endothelial cells.

Peer review

This is a case controlled study demonstrating that plasma but not platelet serotonin levels are increased in patients with liver cirrhosis and correlate with liver insufficiency. The subject is novel and interesting.

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RAPID COMMUNICATION

Preoperative evaluation with T-staging system for hilar cholangiocarcinoma

Ru-Fu Chen, Zhi-Hua Li, Jia-Jia Zhou, Jie Wang, Ji-Sheng Chen, Qing Lin, Qi-Bing Tang, Ning-Fu Peng, Zhi-Peng Jiang, Quan-Bo Zhou

Ru-Fu Chen, Jia-Jia Zhou, Jie Wang, Ji-Sheng Chen, Qing Ling, Qi-Bing Tang, Ning-Fu Peng, Department of Hepatobiliary Surgery, the Affiliated Second Hospital, Sun Yat-sen University, 107 Yan-Jiang Xi Road, GuangZhou 510120, Guangdong Province, China
Zhi-Hua Li, Department of Oncology, the Affiliated Second Hospital, Sun Yat-sen University, 107 Yan-Jiang Xi Road, GuangZhou 510120, Guangdong Province, China
Zhi-Peng Jiang, Department of Emergency, the Affiliated Second Hospital, Sun Yat-sen University, 107 Yan-Jiang Xi Road, GuangZhou 510120, Guangdong Province, China
Supported by Department of Radiology, The Second Affiliated Hospital, Sun Yat-sen University, Guangdong Province, China
Correspondence to: Quan-Bo Zhou, Department of Hepatobiliary Surgery, the Affiliated Second Hospital, Sun Yat-sen University, 107Yan-Jiang Xi Road, GuangZhou 510120, Guangdong Province, China. hnhszqb@yahoo.com.cn
Telephone: +86-13710782185 Fax: +86-20-81332430
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Abstract

AIM: To investigate the clinical value of T-staging system in the preoperative assessment of hilar cholangiocarcinoma.

METHODS: From March 1993 to January 2006, 85 patients who had cholangiocarcinoma diagnosed by operative tissue-biopsy were placed into one of three stages based on the new T-staging system, and it was evaluated the resectability and survival correlated with T-staging.

RESULTS: The likelihood of resection and achieving tumor-free margin decreased progressively with increasing T stage ($P < 0.05$). The cumulative 1-year survival rates of T1, T2 and T3 patients were 71.8%, 50.8% and 12.9% respectively, and the cumulative 3-year survival rate was 34.4%, 18.2% and 0% respectively; the survival of different stage patients differed markedly ($P < 0.001$). Median survival in the hepatic resection group was greater than in the group that did not undergo hepatic resection (28 mo *vs* 18 mo; $P < 0.05$). The overall accuracy for combined MRCP and color Doppler Ultrasonagraphy detecting disease was higher than that of combined using CT and color Doppler Ultrasonagraphy (91.4% *vs* 68%; $P < 0.05$). And it was also higher in detecting port vein involvement (90% *vs* 54.5%; $P < 0.05$).

CONCLUSION: The proposed staging system for hilar cholangiocarcinoma can accurately predict resectability, the likelihood of metastatic disease, and survival. A concomitant partial hepatectomy would help to attain curative resection and the possibility of long-term survival. MRCP/MRA coupled with color Doppler Ultrasonagraphy was necessary for preoperative evaluation of hilar cholangiocarcinoma.

Key words: Hilar cholangiocarcinoma; Preoperative staging; Survival rate; Surgical treatment

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INTRODUCTION

Cholangiocarcinoma is an adenocarcinoma that arises from the bile duct epithelium and is the second most common primary hepatobiliary cancer, however, cholangiocarcinoma remains a relatively rare disease, accounting for < 2% of all human malignancies[1-3]. Although the entire biliary tree is potentially at risk, hilar cholangiocarcinoma which involved the biliary confluence or the right or left hepatic ducts are most common and account for 40%-60% of all cases[4]. In most instances, the prognosis of hilar cholangiocarcinoma is very poor, with an overall 5-years survival rate of only 1%[5]. At present, surgical resection of early detected tumors is still the optimal treatment method for hilar cholangiocarcinoma[6,7]. Therefore, precise preoperative imaging evaluation including classification and staging of tumor is crucial for planning treatment and assessing prognosis.

Currently, the modified Bismuth-Corlette system and American Joint Committee on Cancer (AJCC) systems are still commonly used in evaluation of hilar cholangiocarcinoma in China, but both of them are failure to identify patients who are operative candidates or to provide prognostic information[5,8,9]. It is possible that the AJCC or Bismuth-Corlette systems has been misused by a

generation of medical oncologists and surgeons who have used staging systems based on postoperative evaluation of the tumor to guide the preoperative, intraoperative, and even postoperative management[9,10].

In an attempt to improve the preoperative clinical and prognostic usefulness of stag system, the organizational structure of the hepatobiliary program at Memorial Sloan-Kettering Cancer Center (MSKCC) have proposed a new T staging that takes into consideration both vascular involvement by local tumor extension and the presence or absence of liver atrophy (Table 1)[11]. This proposed T staging system is predictive of resectability, of the likelihood of nodal or distant metastases, and of overall survival[11]. In this study, we used the proposed T staging system, based on imaging data, to stratifies 85 patients hilar cholangiocarcinoma into one of three stages and evaluate the resectability or survival correlated with T-staging. We also want to find the correlation between the T staging and nodal or distant metastases. In addition, in our study, biliary resections coupled with in-continuity hepatic resection has been proposed, to attain radical resection.

Table 1 Revised preoperative T staging system for patients with hilar cholangiocarcinoma

T Stage	Description
T1	Tumor involving biliary confluence ± unilateral extension to 2° biliary radicles No liver atrophy or portal vein involvement
T2	Tumor involving biliary confluence ± unilateral extension to 2° biliary radicles with ipsilateral portal vein involvement ± ipsilateral hepatic lobar atrophy No main portal vein involvement
T3	Tumor involving biliary confluence + bilateral extension to 2° biliary radicles; OR unilateral extension to 2° biliary radicles with controlateral portal vein involvement; OR unilateral extension to 2° biliary radicles with controlateral hepatic lobar atrophy; ralateral hepatic lobar atrophy; phy; OR main or bilateral portal venous involvement

MATERIALS AND METHODS

Data selection

Data was collected from a database of Hepatobiliary Surgery of the Second Affiliated Hospital of SUN Yat-sen University From March 1993 through January 2006, 85 patients of hilar cholangiocarcinoma underwent laparotomy and diagnosed by tissue-biopsy were retrospectively analyzed in this study. There were 45 men and 40 women and their mean age was 63.5 (median, 65; range, 42-81) years. Follow-up was defined as the number of months between the operation date and the date of death or, if the patient was alive, the end dates of the study period (December 31, 2006). Preoperative baseline examinations were electrocardiogram (ECG), sternite, prothrombin time (PT), hepatic and renal function test. All of 85 patients presented with hilar cholangiocarcinoma had more than one kind of image examinations. Ultrasound (US) or duplex ultrasonography (DUS) coupled with tomographic (CT) scanning were performed on 25 patients; duplex ultrasonography coupled with magnetic resonance cholangiopancreatography (MRCP) was performed on 35 patients; ultrasound, CT coupled with ERCP was performed on 11 patients; ultrasound, CT coupled with MRCP was performed on 14 patients.

T-staging

Tumors were restaged retrospectively using revised preoperative T staging system based on preoperative imaging examinations (Table 1). This staging system, a modification of a previously reported scheme[12], classifies tumors according to three factors related to local tumor extent: the location and extent of bile duct involvement (according to the Bismuth-Corlette system)[13,14], the presence or absence of portal venous invasion, and the presence or absence of hepatic lobar atrophy. The survival data was then compared among the stages. T staging correlated with respectability, Ro resection (margins negative), and the incidence of metastatic disease were reviewed.

Surgical strategies

All 85 patients in this study were treated with laparotomy, the type of therapeutic procedures depended on tumor expansion and clinical conditions of patients. If the tumor was resectable, surgery was the first choice of treatment for patients in good clinical conditions. The two types of operations were: (1) local resection of the bile duct alone; (2) extrahepatic biliary resection with in-continuity hepatic resection. In patients with non-resectable tumors or bad clinical conditions, palliative procedure using endoscopic transpapillary and/or percutaneous transhepatic biliary drainage was performed. In this study the survival of all patients undergoing resection of hilar cholangiocarcinomas by either extrahepatic biliary resection alone or by extrahepatic biliary resection with in-continuity hepatic resection were reviewed.

Statistical analysis

All data were analyzed with SPSS 11.5 statistical package. Cumulative overall survival was calculated by the Kaplan-Meier method using the log rank test with T staging. The correlation between T staging and respectability, Ro resection or the incidence of metastatic disease were analyzed using the χ^2 test. Significance was accepted with 95% confidence.

RESULTS

Proposed T-staging system

The follow-up time of all patients was more than 3 mo. Eighty five patients were staged according to the proposed preoperative clinical system, as described above Table 1. Thirty nine patients had tumor involvement of the biliary confluence (with or without unilateral extension to second-order biliary radicles), no portal vein involvement, and no lobar atrophy and were therefore classified as having T1 tumors. Fifteen patients had T2 lesions because of ipsilateral portal vein involvement or ipsilateral lobar atrophy; or both findings. Thirty one patients had T3 tumors because of biliary extent alone, or main portal vein involvement, or metastatic disease.

Table 2 Resectability, incidence of margins negative and metastatic disease after staging by T stage

T Stage	Operative modus			Margins		Metastatic disease	
	Resected	Drainage	Biopsy	Negative	Positive	Negative	Positive
T1 (n = 39)	29 (74.4)	8 (20.5)	2 (5.1)	22 (75.9)	7 (24.1)	24 (61.5)	15 (38.5)
T2 (n = 15)	9 (60.0)	4 (26.7)	2 (13.3)	3 (33.3)	6 (66.7)	7 (46.7)	8 (53.3)
T3 (n = 31)	2 (6.5)	15 (48.4)	14 (45.1)	0 (0)	2 (100)	9 (30.0)	22 (70.0)
χ^2 value		35.5		8.8		7.3	
P value		0.000		0.012		0.026	

The percentages indicate the proportion of patients within each stage grouping or of the total number of patients. Metastatic disease refers to metastases to N2-level lymph nodes or to distant sites. Median survival was calculated for all patients, including those who died perioperatively.

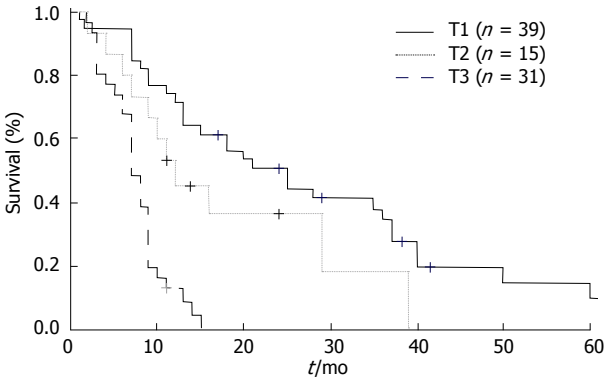

Figure 1 Kaplan-Meier survival analysis stratified by T stage. T stages seemed to be correlated with the survival time (χ^2 = 37.65, P < 0.001), and the survival time decreased progressively with increasing T staging. The cumulative 1-year survival rates of T1, T2 and T3 patients were 71.8%, 50.8% and 12.9%, respectively, and the cumulative 3-year survival rate was 34.4%,18.2% and 0%, respectively, the survival of different stage patients differed markedly (P < 0.001).

The clinical and survival-related factors associated with T stage are detailed in Table 2. Resectability and the likelihood of achieving an R0 resection both decreased progressively with increasing T stage (P < 0.05). A similar proportion of patients with T1 (74.4%) and T2 (60.0%) tumors underwent resection with curative intent. Two patients with T3 tumors (6.5%) were also underwent resection ,but both couldn't achieved R0 resection. In addition, metastatic disease to N2-level lymph nodes or to distant sites (i.e., metastatic disease that contraindicated resection) correlated with increasing T stage (P < 0.05).

Survival analysis

Kaplan-Meier estimate for survival depending on T stages was shown in Figure 1, it showed that T stages seemed to be correlated with the survival time (χ^2 = 37.65, P < 0.001), and the survival time decreased progressively with increasing T staging. The cumulative 1-year survival rates of T1, T2 and T3 patients were 71.8%, 50.8% and 12.9% respectively, and the cumulative 3-years survival rate was 34.4%, 18.2% and 0% respectively; the survival of different stage patients differed markedly (P < 0.001).

In patients with T1 lesions, 12 patients undergoing extrahepatic biliary resections alone and 17 patients underwent in-continuity hepatic resection (Table 3). This is in contrast to patients with T2 lesions, all of patient required a concomitant partial hepatectomy and 3 (33%) of whom required a portal vein resection and reconstruction.

Table 3 Margins negative, complication, operative mortality and survival based on in-continuity hepatic resection

Hepatic resection	n	Margins		Operative mortality, n (%)	Median survival, mo
		Negative, n (%)	Complication, n (%)		
In-continuity Hepatic Resection	28	21 (75)	16 (57)	3 (11)	28
No Hepatic Resection	12	4 (33)	4 (33)	1 (8)	18

Forty of 85 patients underwent resection, 28 (70%) had a concomitant partial hepatectomy, and negative histological margins were attained 75% of the time. The performance of a partial hepatectomy was predictive of a negative histological margin in this series. Median survival in the hepatic resection group was greater than in the group that did not undergo hepatic resection (28 *vs* 18 mo; P < 0.05). And the incident rate of complication and operative mortality in two groups were not different (P > 0.05).

Preoperative imaging evaluation

Most patients were diagnosed after at least a partial radiographic evaluation had been completed, usually consisting in ultrasonography, in a computed tomographic (CT) scan and in some form of direct cholangiography such as ERCP. After referral, further evaluation of tumor extent within the biliary tree and assessment of possible vascular involvement or metastatic disease were performed with MRCP or duplex ultrasonography, which are currently the preferred studies. Some patients who could not be diagnosed preoperatively were considered to have laparotomy for exploration. Altogether, 85 patients had the final diagnosis of hilar cholangiocarcinoma via pathologic diagnosis. The consistent rate with pathological findings of US or duplex ultrasonography coupled with CT, or with MRCP, or with CT and ERCP, or with CT and MRCP was 68% (17/25), 91.4% (32/35), 90.9% (10/11) and 100% (14/14) respectively. And the consistent rate of US combined with CT was significant lower than other combined examinations (P < 0.05). The final diagnosis rate of tumor infiltrated portal vein for duplex ultrasonography combined with MRCP/MRA, duplex ultrasonography combined with CT were 90% (19/21), 54.5% (6/11) respectively. The overall accuracy for combined MRCP and color Doppler Ultrasonagraphy detecting disease and

port vein involvement was higher than that of combined using CT and color Doppler Ultrasonagraphy ($P < 0.05$).

DISCUSSION

Currently, the only curative option for patients with cholangiocarcinoma is aggressive surgical resection. Many authors advocate that patients with suspected cholangiocarcinoma should be considered for operative resection and postoperative adjuvant therapy even if microscopically clear resection margins can not be achieved[15-17]. Therefore, precise preoperative imaging evaluation and staging of tumor are crucial for planning treatment and assessing prognosis.

Great effort has been expended to develop staging systems that are of greater prognostic utility to the surgeon and can be used to guide not only preoperative treatment, but also intra- and postoperative management of patients with cholangiocarcinoma[8,18-20]. There are currently 3 main staging systems utilized for patients with hilar cholangiocarcinomas: AJCC, Bismuth-Corlette, and Blumgart[9]. The AJCC tumor-node-metastasis (TNM) staging system is only applicable to patients who have undergone resection[4]. The Bismuth-Corlette system describes the tumors in terms of their anatomic location. Typically, it has been used to guide treatment (particularly resection), yet it does little to identify patients who are operative candidates or to impart prognostic information[11,12]. The modifications proposed, by Blumgart (Table 1) not only provide anatomic information about the local extent of the tumor but also better stratify patients who are candidates for surgical exploration by taking into account parenchymal characteristics of the liver.

In this study, we retrospectively reviewed 85 patients with cholangiocarcinoma who underwent resection and restaged them into the T-staging system. We have demonstrated the correlation between resectability or R0 resection or survival time and T stage, in 85 patients with hilar cholangiocarcinoma. Resectability and the likelihood of achieving an R0 resection both decreased progressively with increasing T stage. Patients with T1 and T2 have a chance of R0 resection; T3 stage tumors usually have no chance for resection. In our data, a similar proportion of patients with T1 (74.4%) and T2 (60.0%) tumors underwent resection with curative intent. Two patients with T3 tumors (6.5%) also underwent resection, but both couldn't achieve R0 resection. Kaplan-Meier analysis revealed that T-staging seemed to correlate with survival time, and the 1, 3-years accumulative survival time decreased with increasing T stage. The media survival time of patients with T1 stage was significantly higher than that of patients with T2 or T3 stage. In addition, metastatic disease to N2-level lymph nodes or to distant sites (i.e., metastatic disease that contraindicated resection) correlated with increasing T stage. In order to improve operative resectability and curability, hepatic resection has been applied to the treatment for hilar cholangiocar cinoma[21-23]. Whereas, several authors reported that the extent of hepatic resection was closely associated with the occurrence of postoperative complications, such as liver failure, sepsis, and anastomosis leakage[22,24-27]. In this study, we demonstrated a significant survival benefit in those patients who were able to undergo extrahepatic biliary resections coupled with in-continuity hepatic resection as compared with those undergoing to extrahepatic biliary resections alone. Hepatic resection did not increase the incurrence of complication and operative mortality in this series; this results also were supported by Miyazaki *et al*[28]. In our experience, the segments I and Ⅳ resection for hilar cholangiocarcinoma have the benefit of preserving enough hepatic mass for the patient to tolerate surgical stress as compared with major hepatic resection.

Early detection and accurate staging are crucial for planning treatment and improving survival rate of hilar cholangiocarcinomas. Noninvasive methods like magnetic resonance cholangiopancreatography or magnetic resonance angiography (MRCP/MRA) and Doppler ultrasound have been proposed by Jarnagin[29]. In our data analysis, duplex ultrasonography and magnetic resonance cholangiopancreatography have been successful when compared with any other combination of examinations. Ultrasonagraphy detecting disease and port vein involvement was 91.4% and 90%.and was higher than that of combined using CT and color Doppler Ultrasonagraphy. Duplex ultrasonography is noninvasive, and a skilled operator can identify the site of biliary obstruction, as well as the presence or absence of portal venous involvement. The efficacy of MRCP/MRA as a noninvasive means of acquiring reliable and precise information about the anatomy of both the intrahepatic and the extrahepatic biliary tree, as well as the level of tumor involvement, and the presence of nodal or distant metastases, has been well documented and has all but replaced percutaneous and endoscopic cholangiography[30-32].

In conclusion, the T-staging system correlates with respectability, RO resection and overall accumulative survival. Patients with hilar cholangiocarcinoma in T1 and T2 stage have the chance of curative resection, in-continuity hepatic resection, and it is necessary to achieve this with lower complications. Duplex ultrasonography and MRCP/MRA are essential for a preoperative assessment of hilar cholangiocarcinoma.

COMMENTS

Background

Cholangiocarcinoma is a malignancy with poor prognosis, and the best result still comes from surgical resection. However, in many of patients who underwent laparotomy the tumors are found not respectable. Therefore, a precise preoperative evaluation system seems to be particularly important. Whereas, currently the modified Bismuth-Corlette system and American Joint Committee on Cancer (AJCC) systems are still commonly used in evaluation of hilar cholangiocarcinoma in China, but both of them failed to identify patients who are operative candidates or to provide prognostic information.

Research frontiers

This current study retrospectively analyzes 85 patients with cholangiocarcinoma who underwent surgery using the T-staging system, and evaluates whether the resectability or survival correlated with T-staging. We also wanted to find the correlation between the T staging and nodal or distant metastases. Additionally, in our study, biliary resections coupled with in-continuity hepatic resection has been proposed to attain radical resection.

Innovations and breakthroughs

In our data, a similar proportion of patients with T1 (74.4%) and T2 (60.0%) tumors underwent resection with curative intent. Hepatic resection has been applied to the treatment for hilar cholangiocarcinoma, and achieved higher median survival than in the group that did not undergo hepatic resection (28 *vs* 18 mo; $P < 0.05$). In our study, the segments I and IV resection for hilar cholangiocarcinoma have the benefit of preserving enough hepatic mass for the patient to tolerate surgical stress as compared with major hepatic resection. In our data analysis, duplex ultrasonography and magnetic resonance cholangiopancreatography are chosen over any other combination of examinations.

Applications

This study provided important reference as regard to the preoperative assessment for patients with halir cholangiocarcinoma. In conclusion, the T-staging system correlates with resectability, RO resection and overall accumulative survival. Patients with hilar cholangiocarcinoma in T1 and T2 stages have the chance of curative resection, and in-continuity hepatic resection is necessary to achieve this with lower complications. Duplex ultrasonography and MRCP/MRA are necessary for a preoperative assessment of hilar cholangiocarcinoma.

Terminology

In current study, revised preoperative T staging systems were used to preoperatively evaluate the status of patients with hilar cholangiocarcinoma, and it described as follows: T1, Tumor involving biliary confluence ± unilateral extension to 2° biliary radicles, No liver atrophy or portal vein involvement. T2, Tumor involving biliary confluence ± unilateral extension to 2° biliary radicles with ipsilateral portal vein involvement ± ipsilateral hepatic lobar atrophy, No main portal vein involvement T3, Tumor involving biliary confluence + bilateral extension to 2° biliary radicles; OR unilateral extension to 2° biliary radicles with controlateral portal vein involvement; OR unilateral extension to 2° biliary radicles with controlateral hepatic lobar atrophy; OR main or bilateral portal venous involvement.

Peer review

The manuscript retrospectively analyzes 85 patients with cholangiocarcinoma who underwent surgery using the T-staging system. The results show resectability, tumor-free margin resection and cumulative survival of patients decrease with increasing T stage. The study also suggests that MRCP/MRA coupled with Doppler Ultrasonagraphy provides better preoperative evaluation of hilar cholangiocarcinoma. The paper is well written and more importantly it does provide important reference as regard to the preoperative assessment for patient with halir cholangiocarcinoma.

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RAPID COMMUNICATION

Genetic polymorphisms of *ADH2* and *ALDH2* association with esophageal cancer risk in southwest China

Shu-Juan Yang, Hua-Yu Wang, Xiao-Qing Li, Hui-Zhang Du, Can-Jie Zheng, Huai-Gong Chen, Xiao-Yan Mu, Chun-Xia Yang

Shu-Juan Yang, Xiao-Qing Li, Can-Jie Zheng, Huai-Gong Chen, Chun-Xia Yang, Department of Epidemiology, Huaxi Public Health School, Sichuan University, Chengdu 610041, Sichuan Province, China
Hua-Yu Wang, Hui-Zhang Du, Xiao-Yan Mu, Yanting Cancer Prevention and Treatment Institute. Yanting 621600, Sichuan Province, China
Supported partly by Sichuan University Youth Scientific Research Fund (2003) and partly by research fund from Sasakawa Classmate Association (065)
Correspondence to: Dr. Chun-Xia Yang, Department of Epidemiology, Huaxi Public Health School, Sichuan University, Chengdu 610041, Sichuan Province, China. chunxia815@yahoo.com.cn
Telephone: +86-28-85501604 Fax: +86-28-85501295
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Abstract

AIM: To evaluate the impact of alcohol dehydrogenase *2 (ADH2)* and aldehyde dehydrogenase *2 (ALDH2)* polymorphisms on esophageal cancer risk.

METHODS: One hundred and ninety-one esophageal cancer patients and 198 healthy controls from Yanting County were enrolled in this study. *ADH2* and *ALDH2* genotypes were examined by polymerase-chain-reaction with the confronting-two-pair-primer (PCR-CTPP) method. Unconditional logistic regression was used to calculate the odds ratios (OR) and 95% confidence interval (95% CI).

RESULTS: Both *ADH2*1* allele and *ALDH2*1/*2* allele showed an increased risk of developing esophageal cancer. The adjusted OR (95% CI) for *ADH2*1* allele compared with *ADH2*2/*2* was 1.65 (95% CI = 1.02-2.68) and 1.67 (95% CI = 1.02-2.72) for *ALDH2*1/*2* compared with *ALDH2*1/*1*. A significant interaction between *ALDH2* and drinking was detected regarding esophageal cancer risk, the OR was 1.83 (95% CI = 1.13-2.95). Furthermore, when compared with *ADH2*2/*2* and *ALDH2*1/*1* carriers, *ADH2*1* and *ALDH2*2* carriers showed an elevated risk of developing esophageal cancer among non-alcohol drinkers (OR = 2.46, 95% CI = 0.98-6.14), and a significantly elevated risk of developing esophageal cancer among alcohol drinkers among alcohol drinkers (OR = 9.86, 95% CI = 3.10-31.38).

CONCLUSION: *ADH2* and *ALDH2* genotypes are associated with esophageal cancer risk. *ADH2*1* allele and *ALDH2*2* allele carriers have a much higher risk of developing esophageal cancer, especially among alcohol drinkers.

Key words: Esophageal cancer; Alcohol dehydrogenase 2; Aldehyde dehydrogenase 2; Genetic polymorphisms

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INTRODUCTION

Epidemiological studies have consistently shown that alcohol drinking is a strong risk factor for esophageal cancer[1-3]. Alcohol is not a carcinogen, but its primary metabolite, acetaldehyde, has been proven carcinogenic in experimental models[4,5]. When consumed through drinking, ethanol is metabolized primarily by class I alcohol dehydrogenase (ADH2) into acetaldehyde, an intermediate metabolite, followed by aldehyde dehydrogenase (ALDH2) into acetic acid in humans[6]. Acetaldehyde, a well-known carcinogen in animals, plays an important role in alcohol toxicity to humans[7]. The encoding genes of the two representative alcohol-metabolizing enzymes display polymorphisms which may modulate individual differences in alcohol-oxidizing capacity and drinking behavior[7,8]. *ADH2*2/*2* has about 40 times higher Vmax than the less-active *ADH2*1/*1*. *ALDH2*2* allele encodes a catalytically inactive subunit for the ALDH2 polymorphism[6,9]. Individuals with the *ALDH2*1/*2* genotype have only 6.25% of normal *ALDH2 *1/*1* activity, indicating a dominant effect of *ALDH2*2*[10]. *ADH2*2* allele and *ALDH2*2* allele, both leading to high acetaldehyde concentrations, are clustered in East Asian populations[6,11,12]. Therefore, polymorphisms of these two genes may exert their effects on esophageal cancer susceptibility. Although several studies on *ADH2* or *ALDH2* polymorphisms and esophageal cancer risk have been conducted to clarify their association[13-15], investigations on non-alcoholic drinkers are limited[16-19].

Yanting, a rural county of Sichuan Province, is one of the areas with the highest esophageal cancer mortality in China[20]. According to the report from Tumor Registry of China, the average incidence rate in this area was $100.5/10^5$ for males and $76.5/10^5$ for females during 1999-2003, which was higher than that in Linxian County and lower than that in Cixian County of Hebei Province, China. Our previous study in Yanting County has shown that alcohol drinking and smoking are common in Yanting County and the main contributors to esophageal cancer[21]. To further study alcohol-related gene polymorphisms and gene-environment interaction on esophageal cancer, a case-control study was conducted in Yanting County.

MATERIALS AND METHODS

Subjects

Esophageal cancer patients were consecutively collected from the Hospital of Yangting Cancer Research Institute (YCRI) from July 2003 to July 2004. All the patients having lived in Yanting County for more than five years were histologically diagnosed as esophageal cancer within 6 mo at the age of 35-85 years. A total of 191 patients (183 with squamous cell carcinoma and 8 with adenocarcinoma) were recruited for the study. One hundred and ninety-one healthy residents from Yanting County served as controls. In total, 191 patients and 198 controls completed a questionnaire and each provided 1 mL blood. The questionnaire included basic demographic data, information on esophageal cancer and habits such as smoking and alcohol drinking, as well as information on food and nutrition.

The ethics committee of each collaborating institution reviewed and approved the study, and informed consent was obtained from all participants.

Genotyping of ALDH2 and ADH2

Genotyping was based upon duplex polymerase-chain-reaction with the confronting-two-pair-primer (PCR-CTPP) method[7]. Briefly, the sequences of four primers used for ADH2 polymorphism are F1 ADH2: 5'-GGG CTTTAGACTGAATAACCTTGG-3'; R1 ADH2: 5'-AAC CACGTGGTCATCTGTGC-3'; F2 ADH2: 5'-GGTGGC TGTAGGAATCTGTCA-5'; R2 ADH2: 5'-AGGGAA AGAGGAAACTCCTGAA-3'. The sequences of primers used for ALDH2 polymorphism are F1 ALDH2: 5'-TGC TATGATGTGTTTGGAGCC-3'; R1 ALDH2: 5'-CCC ACACTCACAGTTTTCACTTC-3'; F2 ALDH2: 5'-GGG CTGCAGGCATACACTA-3'; R2 ALDH2: 5'-GGC TCCGAGCCACCA-3'. Each 25 μL reaction mixture contained 1.3 U Tag biocatalysts, 1.8 mmol/L Mg^{2+}, 0.24 mmol/L dNTPs, 8 primers, 15 pmol of each primer and 5-8 μL template. The PCR conditions were as follows: initial denaturation at 94℃ for 5 min, followed by 35 cycles at 94℃ for 65 s, at 60℃ for 65 s, at 72℃ for 90 s, and a final extension at 72℃ for 5 min. After transient centrifugation, agarose electrophoresis was conducted. The PCR products included 119 bp fragments of *ALDH2*1* allele, 98 bp fragments of *ALDH2*2* allele, 219 bp fragments of *ADH2* and *ADH2*1* allele, 280bp fragments of *ADH2*2* allele. The 176 bp and 219 bp fragments were the common fragments of the two alleles.

Table 1 Characteristics of patients and controls *n* (%)

Characteristic	Patients *n* = 191	Controls *n* = 198	*P*[1]	OR (95% CI)[2]
Age (yr)				
< 50	28 (14.7)	84 (42.9)		
50-64	118 (61.7)	67 (33.4)	*P* < 0.001	-
⩾ 65	45 (23.6)	47 (23.7)		
Mean age (SD)	58.3 (8.3)	52.8 (13.2)	-	-
Sex				
Male	126 (66.0)	122 (61.6)	*P* = 0.372	-
Female	65 (34.0)	76 (38.4)		-
Smoking status				
Non-smokers[3]	75 (39.3)	121 (61.1)	*P* < 0.001	1.00 (References)
Current smokers	116 (60.7)	77 (38.9)		3.76 (2.01-6.73)
Alcohol drinking status				
Non-drinkers[4]	80 (41.9)	128 (64.7)		1.00 (References)
Current alcohol drinkers	111 (58.1)	70 (35.3)	*P* < 0.001	3.16 (1.91-5.24)

[1]*P* value by chi-square test; [2]ORs for smoking and drinking were adjusted for age and sex, rapid food eating, quality of drinking water, consumption of fresh fruits, vegetable and eggs; [3]Non-smokers including ex-smokers; [4]Non-drinkers including ex-drinkers.

Statistical analysis

Statistical analyses were performed using the STATA statistical package (version 8, STATA, College Station, TX). Demographic data, smoking and drinking status were compared between patients and controls by chi-square test. The subjects smoking more than 10 cigarettes per week for at least 6 mo were defined as current smokers. The subjects consuming more than 50 mL of distilled spirits per week for at least 6 mo were defined as current drinkers. The odds ratio (OR) and 95% confidence interval (95% CI) generated in unconditional logistic regression model were used as measures of association for the risk of esophageal cancer. The relationship of ALDH2 and ADH2 polymorphisms with esophageal cancer risk was determined after adjustment for sex, age, smoking, rapid food eating, quality of drinking water, consumption of fresh fruits, vegetables and eggs. The combined effect of alcohol consumption and *ADH2* and *ALDH2* genotypes on esophageal cancer was also examined in this study. Chi-square test was used to check the Hardy-Weinberg equilibrium (HWE) in controls for the assessment of discrepancies between genotype and allele frequencies.

RESULTS

The characteristics of subjects are listed in Table 1. The mean age of 191 patients and 198 controls was 58.3 and 52.8 years, respectively. There was a significant difference in smoking and alcohol drinking status ($P < 0.001$) between patients and controls. When compared with non-smokers, the adjusted OR of current smokers was 3.76 (95% CI = 2.01-6.73). Current alcohol drinkers also showed an increased risk of developing esophageal cancer (OR = 3.16, 95% CI = 1.91-5.24) when compared with non-drinkers. Almost all the alcohol drinkers drank hard

Table 2 *ADH2, ALDH2* **genotype and allele frequencies and ORs for esophageal cancer** *n* **(%)**

ADH2	Cases (*n* = 191)	Controls (*n* = 198)	OR (95% CI)[1]
**2/*2*	78 (40.8)	100 (50.5)	1.00 (Reference)
**1/*2*	80 (41.9)	76 (38.4)	1.89 (1.10-3.22)
**1/*1*	33 (17.3)	22 (11.1)	1.91 (0.92-3.95)
**1/*2 + *1/*1*	113 (59.2)	98 (49.5)	1.65 (1.02-2.68)
ALDH2			
**1/*1*	90 (47.1)	108 (54.5)	1.00 (References)
**1/*2*	98 (51.3)	76 (38.4)	1.67 (1.02-2.72)
**2/*2*	3 (1.6)	14 (7.1)	0.26 (0.06-1.09)
**1/*2 + *2/*2*	101 (52.9)	90 (45.5)	1.43 (0.89-2.30)
Allele frequencies			OR (95% CI)[2]
ADH2			
**2*	236 (61.8)	276 (69.7)	1.00 (References)
**1*	146 (38.2)	120 (30.3)	1.42 (1.06-1.92)
ALDH2			
**1*	278 (72.8)	292 (73.7)	1.00 (References)
**2*	104 (27.2)	104 (26.3)	1.05 (0.76-1.44)

[1]ORs for gene frequencies were adjusted for sex, age, smoking, drinking, rapid food eating, quality of drinking water, consumption of fresh fruits, vegetable and eggs; [2]ORs for allele frequencies were not adjusted.

liquor containing over 48% ethanol in this area and the main tobacco type was cigarette (data not shown).

The genotype and allele distribution of *ADH2* and *ALDH2* and their OR (95% CI) for esophageal cancer risk are listed in Table 2. The ADH2 genotype frequency was 50.5% for *ADH2*2/*2*, 38.4% for *ADH2*1/*2*, and 11.1% for *ADH2*1/*1* in controls, which were in accordance with the HWE (P = 0.20) (data not shown). When compared with the *ADH2*2/*2* genotype, the esophageal cancer risk in subjects harboring *ADH2*1* allele was significantly elevated (OR = 1.65, 95% CI = 1.02-2.68). The OR for *ADH2*1* allele carriers was 1.42 (95% CI = 1.06-1.92) when compared with *ADH2*2* allele carriers. The frequency of *ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* was 54.6%, 38.4% and 7.1%, respectively, in controls, which were also in accordance with the HWE (P = 0.90) (data not shown). The *ALDH2*1/*2* genotype was associated with an increased risk of developing esophageal cancer (OR = 1.67, 95% CI = 1.02-2.72).

The combined effect of alcohol consumption and *ADH2* and *ALDH2* genotypes on esophageal cancer risk is shown in Table 3. When compared with non-drinkers harboring *ADH2*2/*2* genotype, alcohol drinkers carrying *ADH2*1* showed an increased risk of developing esophageal cancer (OR = 3.94, 95% CI = 1.76-8.81). Similarly, a significantly increased risk of developing esophageal cancer was found in alcohol drinkers harboring *ALDH2*2* genotype (OR = 4.82, 95% CI = 2.06-11.27), compared with non-drinkers harboring *ALDH2*1/*1* genotype. Furthermore, a significant interaction between *ALDH2* and alcohol drinking was detected regarding esophageal cancer risk (adjusted OR = 1.83, 95% CI = 1.13-2.95) (data not shown). When compared with *ADH2*2/*2* and *ALDH2*1/*1* carriers, *ADH2*1* and *ALDH2*2* carriers showed an elevated risk of developing esophageal cancer in non-drinkers (OR = 2.46, 95% CI = 0.98-6.14) and a significantly elevated risk of developing esophageal cancer in alcohol drinkers (OR = 9.86, 95% CI = 3.10-31.38).

DISCUSSION

In the present study, the risk of developing esophageal cancer was significantly increased in *ALDH2*1/*2* gene carriers; subjects with *ADH2*1* allele had a higher risk of developing esophageal cancer than those with *ADH2*2/*2*; carrying both *ALDH2*2* allele and *ADH2*1* allele, suggesting that alcohol drinking greatly increases the susceptibility to esophageal cancer.

*ALDH2*2* allele encoding an inactive subunit of ALDH2 is prevalent in Asian[22]. It was reported that acetaldehyde concentrations after drinking alcohol are mainly dependent on the enzyme activation of *ALDH2*[6,23]. After consumption of alcohol, blood acetaldehyde concentrations in those with *ALDH2*2/*2* and *ALDH2*1/*2* are 19- and 6-fold higher than in those with *ALDH2*1/*1*[24]. Case-control studies of Japanese and Chinese alcohol drinkers[10,15,16,17,19,25,26] consistently demonstrated that inactive *ALDH2*1/*2* is a strong risk factor for esophageal cancer. Our data also show that individuals with *ALDH2*1/*2* (OR = 1.67, 95% CI = 1.02-2.72) had a significantly increased risk of developing esophageal cancer compared to those with *ALDH2*1/*1*. Furthermore, our results reveal that there was a significant interaction between *ALDH2* and alcohol drinking, indicating that esophageal cancer is associated with alcohol drinking, which is influenced by the polymorphism of *ALDH2*.

Previous case-control studies investigating the association between *ADH2* genotype and esophageal cancer demonstrated that *ADH2*1* allele independently enhances esophageal cancer risk[6,10,14,17,19]. In our study, the adjusted OR for subjects carrying *ADH2*1* allele was 1.65 (95% CI = 1.02-2.68), which is in line with former studies[6,10,14,17,19]. There are several reasons which may explain this finding. ADH2 is the predominant enzyme among low-Km class I ADHs expressed in the esophagus[27]. In *ADH2*1/*1* homozygotes, concentrations of ethanol may linger in the esophageal mucosa during the slow oxidation of *ADH2*. Although ethanol is not a cancerogen itself, tobaccos and some other exogenous cancerogens would be assimilated much easier, thus increasing the effect of cancerogen. Besides, ethanol can induce the composition of phase I drug metabolism enzymes such as CYP2E1. Moreover, alcohol drinkers with *ADH2*1* genotype tend to have experienced 'binge-drinking' and withdrawal syndrome earlier in life than those with other genotypes[14,23]. Therefore, *ADH2*1*-mediated alcohol-related events may contribute to the enhancement of esophageal cancer risk in alcohol drinkers.

It was reported that combination of *ADH2*1* allele and *ALDH2*2* allele can greatly enhance cancer risk among alcoholics[10] and general populations[6,19,23]. Carrying these two alleles simultaneously indicates a longer time of exposure to alcohol and highly-concentrated acetaldehyde, thus increasing the individual's susceptibility to esophageal cancer. In the present study, the OR for alcohol drinkers

Table 3 Combined effect of alcohol consumption and *ADH2* and *ALDH2* polymorphisms on esophageal cancer *n* (%)

ADH2		Alcohol drinking status					
		Non-drinkers[2]			Current alcohol drinkers		
		Cases (*n* = 80)	Controls (*n* = 128)	OR (95% CI)[1]	Cases (*n* = 111)	Controls (*n* = 70)	OR (95% CI)[1]
*2/*2		37 (46.3)	65 (50.8)	1.00 (References)	41 (36.9)	35 (50.0)	1.88 (0.86-4.15)
*1/*2 or *1/*1		43 (53.7)	63 (49.2)	1.21 (0.63-2.33)	70 (63.1)	35 (50.0)	3.94 (1.76-8.81)
ALDH2							
*1/*1		33 (41.3)	67 (52.3)	1.00 (References)	57 (51.4)	41 (58.6)	3.15 (1.39-7.13)
*1/*2 or *2/*2		47 (58.7)	61 (47.7)	2.03 (1.03-3.99)	54 (48.6)	29 (41.4)	4.82 (2.06-11.27)
ADH2 and ALDH2							
*2/*2	*1/*1	15 (18.8)	42 (32.8)	1.00 (References)	17 (15.3)	20 (28.6)	2.54 (0.84-7.67)
*1/*2 or *1/*1	*1/*2 or *2/*2	25 (31.3)	38 (29.7)	2.46 (0.98-6.14)	30 (27.0)	14 (20.0)	9.86 (3.10-31.38)

[1]ORs were adjusted for sex, age, smoking, rapid food eating, quality of drinking water, consumption of picked vegetables and fresh fruits, vegetables and eggs; [2]Non-drinkers including ex-drinkers.

with both *ADH2*1* allele and *ALDH2*2* allele was 9.86 (95% CI = 3.10-31.38), which is consistent with former studies[10].

Some limitations of this study should be considered. One is that controls were selected from residents in Yanting County and their basic features are consistent with general people, such as smoking and alcohol drinking. In this study, the genotype distribution among the controls closely conformed to the Hardy-Weinberg equilibrium. So our control group represents the general population of Yanting County. In addition, the present study was not an age matched case control study and age is a risk factor for esophageal cancer. However, our results are age-adjusted and may not be biased by age. The small number of subjects is another limitation, so further studies in a larger scale appear warranted.

In conclusion, *ADH2* and *ALDH2* genotypes are associated with esophageal cancer risk. In addition, the risk of developing esophageal cancer increases in subjects carrying *ADH2*1* allele and *ALDH2*1* allele, especially in alcohol drinkers. Our present findings provide more information on the *ADH2* and *ALDH2* polymorphisms of esophageal cancer in Chinese.

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COMMENTS

Background

Epidemiological studies have consistently shown that alcohol drinking is a strong risk factor for esophageal cancer. When consumed through drinking, ethanol is metabolized primarily by class I alcohol dehydrogenase (ADH2) into acetaldehyde, an intermediate metabolite, followed by aldehyde dehydrogenase (ALDH2) into acetic acid in humans. Acetaldehyde, a well-known carcinogen in animals, plays an important role in alcohol toxicity to humans. *ADH2*2* allele and *ALDH2*2* allele, both leading to high acetaldehyde concentrations, are clustered in East Asian populations. Therefore, the polymorphism of these two genes may exert effect on esophageal cancer susceptibility.

Research frontiers

Esophageal cancer risk is associated with habits and food consumption, such as smoking, alcohol drinking, consumption of fresh fruits and vegetables. However, the effect of gene polymorphisms or gene-environment interaction on esophageal cancer risk has become a hotspot in recent researches. The present study reports the association of ADH2 and ALDH2 gene polymorphisms with esophageal cancer risk. Furthermore, interaction and combination impacts on esophageal cancer risk between gene polymorphisms and alcohol drinking are also analyzed and discussed.

Innovations and breakthroughs

Our study showed that *ADH2* and *ALDH2* polymorphisms were associated with esophageal cancer risk in a high-incidence area of southwest China. Previous studies were mainly conducted in Japanese males or alcoholics. In addition, our controls were collected from the healthy residents and the patients were histologically diagnosed as esophageal cancer within 6 mo, which is superior to hospital-based case-control studies.

Applications

The present study indicates ADH2 and ALDH2 genotypes are associated with esophageal cancer risk, the risk of developing esophageal cancer increases in subjects carrying ADH2*1 allele and ALDH2*2 allele, especially in alcohol drinkers. The present findings can provide more information on the ADH2 and ALDH2 polymorphisms of esophageal cancer in Chinese and help make the prevention strategy against esophageal cancer in China.

Terminology

Alcohol dehydrogenase 2 (*ADH2*): A zinc-containing enzyme which oxidizes primary and secondary alcohols or hemiacetals in the presence of NAD. In alcoholic fermentation, it catalyzes the final step of reducing aldehyde to alcohol in the presence of NADH and hydrogen. Dehydrogenase 2 (*ALDH2*): An enzyme that oxidizes aldehyde in the presence of NAD+ and water to acid and NADH. Genetic polymorphisms: The regular and simultaneous occurrence of two or more discontinuous genotypes in a single interbreeding population. The concept includes differences in genotypes ranging in size from a single nucleotide site to large nucleotide sequences visible at a chromosomal level.

Peer review

This is an interesting paper investigating the association between ADH2 and ALDH2 polymorphisms, environmental factors and esophageal cancer risk in a relatively small cohort of cancer patients. The main conclusion of the manuscript is that ADH2 and ALDH2 genotypes are associated with esophageal cancer risk; the risk of developing esophageal cancer increases greatly in subjects carrying ADH2*1 allele and ALDH2*2 allele, especially in alcohol drinkers.

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RAPID COMMUNICATION

Construction of humanized carcinoembryonic antigen specific single chain variable fragment and mitomycin conjugate

De-Jie Chen, Zui Tan, Feng Chen, Tian Du

De-Jie Chen, Zui Tan, Feng Chen, Tian Du, Department of General Surgery, Zhongnan Hospital of Wuhan University, Wuhan 430071, Hubei Province, China
Correspondence to: Professor Zui Tan, Department of General Surgery, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan 430071, Hubei Province, China. docdj_1130@yahoo.com.cn
Telephone: +86-27-63436739
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Abstract

AIM: To construct a new target-oriented conjugate of humanized carcinoembryonic antigen (CEA) specific single chain variable fragment (scFv) and mitomycin (MMC) against colorectal cancer, and to investigate its influence on the growth and apoptosis of colorectal cancer cells.

METHODS: The primer was designed according to the gene sequence described in reference 16, which respectively contains restriction enzyme cleavage sites *Bam*H Ⅰ and *Eco*R Ⅰ in its upstream and downstream. PCR was performed with the plasmid as template containing genes of humanized anti-CEA scFv. The product was digested by *Bam*H Ⅰ and *Eco*R Ⅰ, and connected to an expression vector which also has the restriction enzyme cleavage sites *Bam*H Ⅰ and *Eco*R. Expression of the reaction was induced by isopropy-β-D-thiogalactoside (IPTG). Then the expression product was covalently coupled with MMC by dextran T-40. The immunoreactivity of the conjugate against colorectal cancer cells as well as CEA was measured by enzyme linked immunosorbent assay (ELISA). The inhibiting ratio of conjugate on the growth of colorectal cancer cells was also measured by ELISA. The effect of conjugate on the apoptosis of colorectal cancer cells was determined by flow cytometry (FCM).

RESULTS: Restriction endonuclease cleavage and gene sequencing confirmed that the expression vector was successfully constructed. Sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) confirmed that this vector correctly expressed the fusion protein. ELISA confirmed that the conjugate had quite a strong immunoreactivity against colorectal cancer cells and CEA. The conjugate had inhibitory effects on colorectal cancer cells in a concentration-dependent manner and could induce apoptosis of colorectal cancer cells in a concentration-dependent manner.

CONCLUSION: The CEA-scFv-MMC conjugate can be successfully constructed and is able to inhibit the growth and induce apoptosis of colorectal cancer cells.

Key words: Carcinoembryonic antigen; Single chain variable fragment; Mitomycin; Immunoconjugates; Colorectal cancer

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INTRODUCTION

Significant improvement has been achieved in the treatment of advanced colorectal cancer in the past decade[1]. New cytotoxic agents and new monoclonal antibodies (mAb) have been shown to substantially improve patient outcomes in randomized trials[2]. Nevertheless, the prognosis of patients with advanced colorectal cancer remains relatively poor with a median survival of approximately 20 mo in optimally treated patients[3]. Therefore, additional treatment strategies are needed in order to further improve the outcomes.

With advances in oncomolecular biology, the mechanism of tumor genesis and development is better understood[4], which provides a new medication pattern against tumors. Since a conception of target-oriented medication against tumors has been recently brought forward[5], we can design and develop medications aiming at target spots of specific molecules and genes to selectively kill tumor cells according to the known abnormal molecules and genes related to the genesis of tumors. Target-oriented medication against tumors provides a new effective way of treating malignant tumors such as mammary cancer, intestinal cancer and lung cancer[6-8], and can directly deliver chemotherapeutics for the tumor and form a high local drug concentration so as to decrease the total dosage and further reduce the toxic and side effects[9].

An ideal target spot of tumor treatment has the following features. (1) It is a kind of macromolecules which are rather critical for malignancy phenotype; (2) It has no obvious expression in important organs and tissues; (3) It has

biological correlativity; (4) It can be repeatedly tested on clinical specimens; (5) It has apparent correlativity with the clinical outcome[10]. Carcinoembryonic antigen (CEA) is a 180 ku cell surface-expressed glycoprotein antigen present in a number of adenocarcinomas, especially in colorectal cancer[11]. The gene sequence and three-dimensional structure of CEA have been reported elsewhere[12]. CEA is a member of the immunoglobulin superfamily and has cell adhesion properties as well as other less clearly defined roles[13]. Since it corresponds to all features of the target spot of tumor treatment, it is the ideal target spot of treating colorectal cancer.

At present, research on target-oriented medication against advanced colorectal cancer involved 3 different target-oriented drugs that have achieved significant curative effect: epidermal growth factor receptor (EGFR) inhibitors, vascular endothelial growth factor receptor (VEGFR) inhibitors, and cyclooxygenase-2 (COX-2) inhibitors[14]. By coupling radio immunity drugs with anti-CEA monoclonal antibody, Vogel[15] has achieved favorable therapeutic effectiveness on nude mice model of liver metastasis of colorectal cancer, thus opening up a new idea for target-oriented medication against advanced colorectal cancer. Based on the above-mentioned theory, we constructed a humanized anti-CEA single-chain antibody (scFv) and coupled it with mitomycin (MMC), a chemotherapeutic agent against colorectal cancer, and investigated the influence of this conjugate on the growth and apoptosis of colorectal cancer cells.

MATERIALS AND METHODS

Materials

Plasmid pUC18 containing humanized anti-CEA-scFv was kindly provided by Professor Mark Sherman (the Berkman Research Institute, USA). *E.coli* DH5α and expression plasmid pGEX-4T-1 were stored in the Virus Research Institute, Medical College of Wuhan University. T4 DNA ligase and restriction endonuclease were purchased from New England Biolabs (USA). High fidelity DNA polymerase and DNA gel extraction kits were purchased from the Promega Company (USA). Plasmid extracting and purifying kits were the products of Vegen Company (Hangzhou, China). Glutathione acyltransferase purification kits were purchased from the Clonetech Company (USA). MMC was purchased from the Roche Company (USA). Bovine serum albumin (BSA), Tween 20, PI dyebath and Hanks solution were bought from Fuzhou Maixin Biology Company (China). Glucosan T-40, cysteine, N-2 N-ethylmaleimide (NEMI), horseradish peroxidase-labeled goat anti-mouse IgG, dimethyl sulphoxide (DMSO) were bought from the Sigma Company (USA). RPMI 1640 culture medium was bought from the Gibico Company (USA). CEA was produced by the Zymed Company (USA). Colorectal cancer cell lines SW480, SW620 and LoVo were provided by China Center for Typical Culture Collection (CCTCC) (Wuhan, China). Primer synthesis and gene sequencing were performed by the Sangon Company (Shanghai, China).

Primers were designed using the program Oligo4.1 and synthesized by Sangon Company (Shanghai, China) as previously described[16], a restriction enzyme cleavage site *Bam*H I was added to the end 5', and a restriction enzyme cleavage site *Eco*R I was added to the end 3'. Upstream primer: 5'CGGGATCCATGGACAGAGTCACA3', downstream primer: 5'CCGAATTCTCCACGTGCACTCGAGACGGTGAC3'. The underlined parts are the restriction enzyme cleavage sites.

Construction of expression vector

Plasmid pUC18 containing humanized anti-CEA scFv T84.66 was taken as the template to perform PCR with its upstream and downstream primers in 50 μL reaction system containing 5 μL template, 5 μL reaction buffer, 2 μL upstream and downstream primers, 0.5 μL high fidelity DNA polymerase, 4 μL dNTP and 33.5 μL deionized water. Samples were heated at 94℃ for 5 min, followed by 35 cycles of heating at 94℃ for 30 s, at 55℃ for 30 s and at 72℃ for 30 s. The temperature was held constant at 72℃ for 7 min to ensure complete extension. The completed PCR reaction mix was electrophoresed on 1% agarose gel and the desired product was extracted from 200 mg gel slice. The purified product was digested with *Bam*H I and *Eco*R. cDNA of anti-CEA scFv T84.66 containing correspondence restriction enzyme sites to pGEX-4T-1 was produced. Prokaryon expression vector pGEX-4T-1 was also digested with *Bam*H I and *Eco*R. After identified by agarose gel electrophoresis, the plasmid with a cohesive end was connected to the cDNA of anti-CEA scFv T84.66 acquired previously. Connection reaction was performed in 10 μL system. The reaction product was transformed into competent cell line DH5α and the positive clone of recombined CEA-scFv-pGEX-4T-1 was acquired. Restriction endonuclease cleavage and gene sequencing confirmed that the scFv fragment was correctly interpolated into pGEX4-T-1. Positive plasmid was extracted and purified with the kits from Vegen Company (Hangzhou, China).

Expression and purification of the fusion protein

Expression of E. coli DH5α containing the positive plasmid was induced by isopropyl-β-D thiogalactoside (IPTG) followed by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE). The level of this protein in total bacterial protein was detected with a thin layer chromatogram (TLC) scanner. The correctly expressed DH5α containing the recombinant plasmid was cultured in 500 mL triangular flask. After IPTG was added to induce expression of the protein, the product was purified with glutathione acyltransferase purification kit according to its manufacturer's instructions. The purified protein was collected with a step-by-step collector containing 500 μL per tube, and the absorbance value was measured at 280 nm to quantify the protein.

Construction of CEA-scFv-MMC conjugate

A pertinent amount of dextran T-40 was admixed with sodium periodate as previously described[17], and the mixture reacted for 3 h at ordinary temperature away from light, then sufficiently dialyzed with deionized water. After cryodesiccation, poly aldehyde dextran (PAD) was produced. Fifty mg of PAD was admixed with 100 mg of MMC, and

the mixture was incubated at 4℃ for 12 h away from light. Then 80 mg of CEA-scFv purified protein was added, stirred reaction was carried out for 12 h and terminated with sodium borohydride. The reaction product was purified with Sephadex G-75 for CEA-scFv-MMC conjugate. The molecular constitution of the conjugate was measured by spectrophotometry. The absorbance value of the conjugate was measured respectively at 280 nm and 488 nm, and at 595 nm after stained with Coomassie brilliant blue. The mole ratio of each composition was recorded.

Detection of immunocompetence of CEA-scFv-MMC conjugate by ELISA

Colorectal tumor cells SW480, SW620 and LoVo were cultured *in vitro* in 96-well culture plates (10^4 cells per well). Twenty-four hours later, 2.5 g/L glutaraldehyde precooled at 4℃ was added (50 μL per well). The cells were fixed at 4℃ for 5min, washed 3 times with PBS and stored at -20℃. When it was used, 10 g/L BSA was added (200 μL per well), sealed overnight at 4℃, then washed with PBS-Tween 20 (PBS-T), and CEA-scFv-MMC diluted with multiple proportion was added (50 μL per well). The mixture was left to react at 37℃ for 1 h, then washed 3 times with PBS-T. Horseradish peroxidase-labeled goat anti-mouse IgG antibody was added (50 μL per well). The mixture was incubated at 37℃ for 1 h, washed 5 times with PBS-T. Enzyme reaction substrate was added (200 μL per well), the mixture was incubated to react for 15 min at room temperature away from light and terminated by adding 2 mol/L sulfuric acid. The absorbance value was measured at 490 nm with enzyme labelling instruments to determine the immunoreactivity of CEA-scFv-MMC against colorectal cancer cells. CEA (pH 7.5) at the concentration of 1.35 g/L was put into culture plates (100 μL per well), the mixture was incubated over night at 4℃. The supernatant was discarded. The mixture was washed 3 times with PBS and stored at -20℃. When it was used, 10 g/L BSA was added (200 μL per well), sealed overnight, and washed with PBS-T buffer solution. Other steps were the same as above, and the immunoreactivity of CEA-scFv-MMC against CEA was detected.

Growth inhibition ratio of conjugate to colorectal cancer cells

The colorectal cancer cells LoVo were cultured in RPMI1640 culture medium, routine serial subculture was carried out in an incubator containing 50 mL/L CO_2 at 37℃. The culture medium was replaced with drug-containing culture medium 24 h after the cells adherently grew to 70%-80% monolayer, then the experiment was carried out. The cells were digested with trypsin and blown to single cell suspension. The cell concentration was counted, adjusted to 1×10^8 cells/L, which were inoculated into 96-well culture plates (0.2 mL per well). The original fluid was discarded after 24 h culture. CEA-scFv-MMC conjugate with different concentrations was added into the wells (0 mg/L in control group; 25 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 300 mg/L and 400 mg/L, respectively, in treatment group) and cultured for 48 h. Twenty μL of MTT solution (2.5 g/L) was added into each well, and cultured for 4 h. After the supernatant was carefully blotted, 150 μL of 100 g/L DMSO was added into each well. The absorbance value (A_{630nm}) of each well was measured by enzyme labelling instruments after gently agitated on the oscillator. The growth inhibition ratio at each concentration in treatment group was calculated according to the following formula: growth inhibition ratio IR (%) = (1-average A_{630nm} of treatment group/A_{630nm} of control group) × 100%.

Influence of conjugate on apoptosis of colorectal cancer cells

Colorectal cancer cells treated with different concentrations of CEA-scFv-MMC conjugate were cultured for 3 d and 10^6 cells were collected, fixed with 700 mL/L ethanol. The dying cell suspension was filtrated through nylon net (400 meshes) and washed 3 times with PBS followed by addition of 10 μL of Annexin V-fluorescein isothiocyanate and PI solution. The mixture was blended in ice bath for 10 min away from light. A flow cytometer (FCM) was used to detect the influence of CEA-scFv-MMC conjugate on the apoptosis of colorectal cancer cells.

Experiments approved by the local ethical committee were performed after the patients gave their informed consent. All the experimental data were expressed as mean ± SD. Comparison between treatment and control groups was made by *t* test and their ratios by χ^2 test. The correlation between the two groups was analyzed with collinearity. $P < 0.05$ was considered statistically significant.

RESULTS

Construction of recombinant plasmid CEA-scFv-pGEX-4T-1

A 810 bp specific band was obtained by PCR amplification with pUC18 as template. cDNA of PCR amplification was completely in accordance with the gene sequence of humanized anti-CEA specific scFv T84.66. The positive clone of recombinant plasmid CEA-scFv-pGEX-4T-1 was identified by double restriction endonuclease cleavage with *Bam*H I and *Eco*R I. Two specific bands were obtained in line 1, one was 4.7 kb and the other was 810 bp, and a 5.5 kb band was obtained in line 2. The 4.7 kb band represented pGEX-4T-1, 810 bp band anti-CEA-scFv, and the 5.5 kb band the recombinant plasmid CEA-scFv-pGEX-4T-1 (Figure 1). The result was completely consistent with our hypothesis. Meanwhile, the scFv gene was correctly inserted to the expression vector.

Expression of scFv gene

After induction of IPTG, expression of the scFv gene inserted to the pGEX-4T-1 was stable. A new protein band was obtained at M_r 49 000 after SDS-PAGE, and its size was in accordance with GST (M_r 26 000) and scFv-CEA (M_r 23 000) fusion protein (Figure 2). This new protein band was preliminarily identified as the expressed CEA-scFv/GST fusion protein. TLC scan showed that the expressed fusion protein amounted to 26% of the total bacterial protein.

Components and immunoreactivity of the conjugate

The components of the conjugate were calculated. The molecule ratio of CEA-scFv: Dextran T-40: MMC in the conjugate was 1:1.2:38. The immunoreactivity of the con-

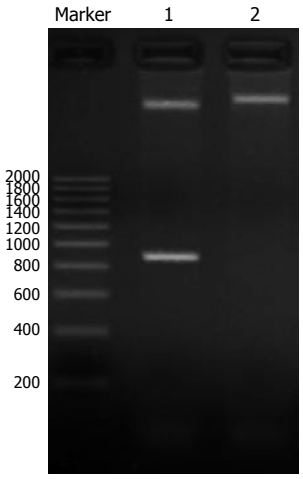

Figure 1 PCR amplification showing 2 specific bands.

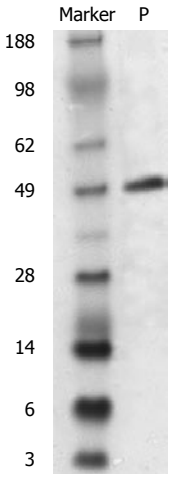

Figure 2 Expression of scFv gene.

jugate against colorectal cancer cells SW480, SW620 and LoVo was strong, and that against LoVo cells was the strongest (Figure 3A). The immunoreactivity of the conjugate against CEA was also strong, especially when the concentration of the conjugate was above 20 mg/L (Figure 3B).

Restraining effect of the conjugate on growth of LoVo cells

After 48 h treatment, LoVo cells were treated with various concentrations of CEA-scFv-MMC, more or less restraining effect of the conjugate on the growth of LoVo cells was shown, significantly depending on the concentration. When the concentration was above 100 mg/L, it had an obvious restraining effect on the growth of the cells ($^aP < 0.05$), and when the concentration was above 200 mg/L, it had a further restraining effect on the cells ($^bP < 0.01$, Table 1).

Effect of conjugate on apoptosis of LoVo cells

Different doses of CEA-scFv-MMC had different effects on apoptosis of LoVo cells. Apoptosis of LoVo cells began at the concentration of 25 mg/L CEA-scFv-MMC. The apoptosis rate increased with the increasing concentration of CEA-scFv-MMC (Figure 4A). Apoptosis of LoVo cells began 12 h after treatment of LoVo cells with CEA-scFv-MMC and the apoptosis rate reached its peak at 72 h (Figure 4B). The results showed that apoptosis of LoVo cells induced by CEA-scFv-MMC was highly dependent on its concentration and its duration of action.

DISCUSSION

Adjuvant chemotherapy has become more and more important in the treatment of colorectal cancer[18]. In recent years, though a variety of anti-cancer drugs are available, most of them could not distinguish cancer cells from normal cells[19]. Therefore, their clinical application is limited due to their toxic and side effects[20]. Target-oriented treatment directly delivers chemotherapeutic drugs to the tumor, resulting in a high drug concentration in the tumor[21]. Through decreasing the total dose, the toxic and side effects of drugs are decreased[22]. Single-chain antibody is an ideal vehicle for delivering chemotherapeutic drugs, because it is easy to reach the tumor due to its small molecular weight and strong penetrating force[23]. The scFv gene applied in this study is derived from monoclonal antibody T84.66, which has been humanized[16] and does not cause (HAMA) reaction in human body[24]. MMC, a broad spectrum anti-tumor drug, is nonspecific for cell cycle. However, it may depolymerize the DNA of cells and inhibit its replication, thus restraining the division of tumor cells[25]. The aim of this study was to construct a drug for target-oriented treatment of colorectal cancer, with MMC as the "warhead" and anti CEA svFv as the vehicle.

Taking plasmid pUC18 as template, we successfully amplified the CEA scFv gene, which is completely consistent with the reported gene sequence[16]. In order to make the fusion gene express effectively and prokaryotic cells express eukaryotic protein, we successfully constructed the GST fusion expressing vector (pGEX-4T-1/CEA-ScFv) and removed the repression effect of lac by IPTG, and made CEA scFv express highly effective in *E.coli* DH5α. The expressed fusion protein amounted to 26% of the total bacterial protein.

In this study, we successfully coupled anti-CEA scFv and MMC with dextran T-40 as a medium. The molecular ratio of scFv: dextran T-40: MMC in the conjugate was 1:1.2:38. Since the molecular weight of the antibody is only 49 ku, the conjugate could meet the requirements of antibody[26]. Measurement of immunoreactivity of the conjugate showed that it had a strong immunological activity against three kinds of colorectal cancer cells, among which immunoreactivity of the conjugate against LoVo cells was the strongest, which may be due to the high CEA expression in LoVo cells[27].

In the study, different concentrations of CEA-scFv-MMC had a different restraining effect on LoVo cells depending on the concentration. When the concentration was above 100 mg/L, it had an obvious restraining effect on the growth of LoVo cells. Apoptosis of LoVo cells began at the concentration of 25 mg/L, the apoptosis ratio increased with the increasing concentration of CEA-scFv-MMC. The optimal dose of CEA-scFv-MMC for inducing apoptosis was 200 mg/L. When time was studied as a variable, the apoptosis began 12 h after treatment of LoVo cells with CEA-scFv-MMC and reached its peak 72 h after CEA-scFv-MMC treatment, suggesting that apoptosis of LoVo cells induced by CEA-scFv-MMC is highly dependent on the concentration of CEA-scFv-MMC and its

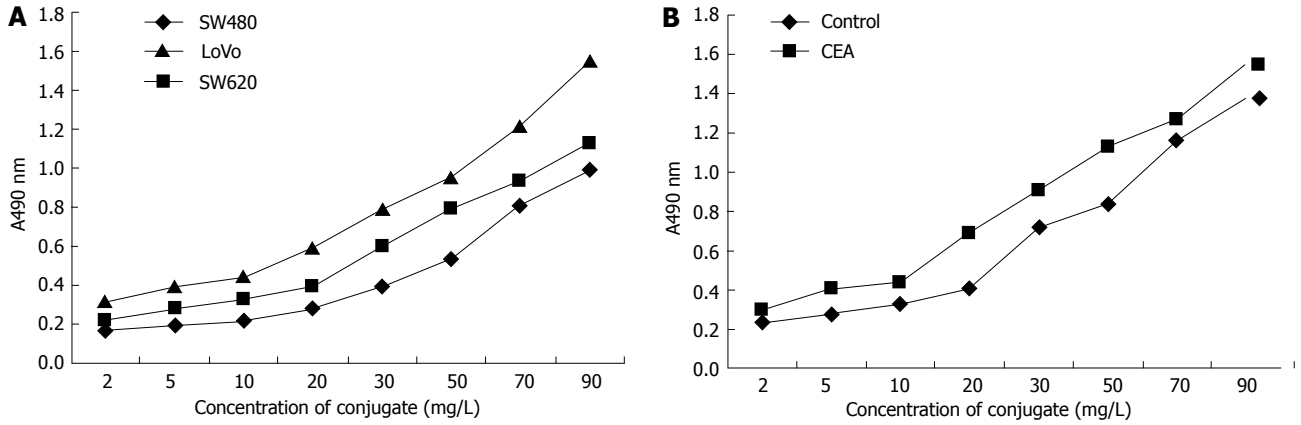

Figure 3 Immunoreactivity of the conjugate against colorectal cancer cells (**A**) and CEA (**B**).

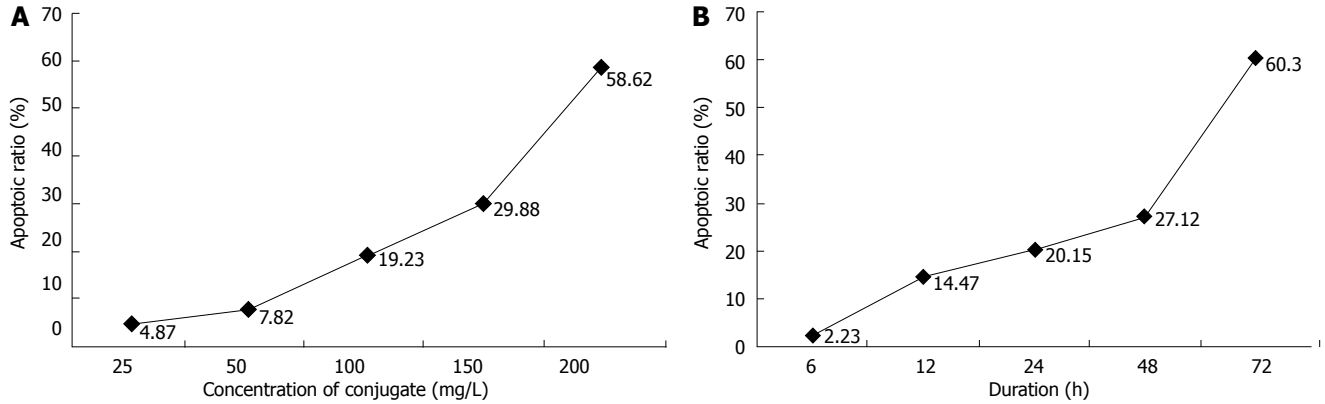

Figure 4 Effect of conjugate on apoptosis of LoVo cells at the concentration of 250 mg/L (**A**) and 200 mg/L (**B**).

duration of action. However, a large dose or a long duration of CEA-scFv-MMC can result in cell necrosis, which may be due to the *in vitro* accumulation of cells undergoing apoptosis without pinocytosis of macrophages[28].

Taking the capacity of the protein yield into consideration, we did not remove GST from the GST/CEA-scFv fusion protein, but directly coupled the fusion protein with MMC. In *in vitro* experiment, GST had no effect on the immune activity of the conjugate and colorectal cancer cells. Further experiments are needed to demonstrate whether the conjugate can be applied *in vivo*. On the other hand, the reaction of the body and immunogenecity do not necessarily parallel the process of humanization[29]. Thus, the in vivo immunogenecity cannot be predicted .The next experiment will focus on more suitable expression vectors to make the expression of CEA-scFv more effectively. Animal experiments will be carried out to explore the target-oriented effect of CEA-scFv-MMC, immunogenecity of the conjugate and its therapeutic effect on colorectal cancer *in vivo*. Since only some kinds of colorectal cancer express CEA[30], this therapy would only be applied to such cancers.

In conclusion, CEA-scFv-MMC conjugate can be successfully constructed and restrains the growth of colorectal cancer cells and induces apoptosis of cancer cells *in vitro*.

Table 1 IR of LoVo cells induced by different concentrations of conjugate

CEA-scFv-MMC (mg/L)	A_{630} (mean ± SD)	Inhibition ratio (%)
Control	0.75 ± 0.10	
25	0.68 ± 0.09	9.3
50	0.62 ± 0.08	17.5
100	0.38 ± 0.06[a]	45.0
150	0.24 ± 0.05[a]	66.9
200	0.11 ± 0.03[b]	84.4
300	0.09 ± 0.02[b]	87.5
400	0.07 ± 0.02[b]	90.0

[a]$P < 0.05$, [b]$P < 0.01$, *vs* control group.

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COMMENTS

Background

Carcinoembryonic antigen (CEA) is present in a number of adenocarcinomas, especially in colorectal cancer. If a humanized anti-CEA single-chain antibody (scFv) can be coupled with a chemotherapeutics, the conjugate would be an ideal target-oriented medication for colorectal cancer.

Research frontiers

An expression vector has been constructed to express the humanized anti-CEA

scFv, with the protein coupled with mitomycin (MMC). The conjugate can restrain the growth of colorectal cancer cells and induce apoptosis of colorectal cancer cells *in vitro*.

Innovations and breakthroughs

Through restriction endonuclease cleavage and gene sequencing, the expression vector was successfully constructed. Applying SDS-PAGE and ELISA, we have confirmed that this vector can correctly express the fusion protein and the conjugate has quite a strong immunoreactivity against colorectal cancer cells and CEA. The conjugate has an inhibitory effect on colorectal cancer cells in a concentration-dependent manner, and induces apoptosis of colorectal cancer cells in a concentration-and time-dependent manner.

Applications

The conjugate may be a potential target-oriented medication for colorectal cancer expressing CEA.

Terminology

CEA is a 180 ku cell-surface expressed glycoprotein antigen present in a number of adenocarcinomas, especially in colorectal cancer. It is a member of the immunoglobulin superfamily and has cell adhesion properties as well as other less clearly defined roles. scFv is an ideal vehicle for delivering chemotherapeutics, as it is easy for single-chain antibody to reach the tumor due to its small molecular weight and strong penetrating force. MMC is a broad spectrum anti-tumor medicine and nonspecific for cell cycle. However, it may depolymerize DNA of cells and inhibit its replication, thus restraining the division of tumor cells.

Peer review

This paper reports the construction and *in vitro* effect of a humanized carcinoembryonic antigen specific single chain fragment mitomycin conjugate. The authors have demonstrated that CEA-scFv-MMC conjugate is able to inhibit the growth and induce the apoptosis of colorectal cancer cells.

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CASE REPORT

Massive gastrointestinal bleeding: An unusual case of asymptomatic extrarenal, visceral, fibromuscular dysplasia

Paula Andrea Rodriguez Urrego, Mark Flanagan, Wilson S Tsai, Craig Rezac, Nicola Barnard

Paula Andrea Rodriguez Urrego, Nicola Barnard, Department of Pathology, Robert Wood Johnson Medical School-University of Medicine and Dentistry of New Jersey, United States
Mark Flanagan, Craig Rezac, Department of Surgery, RWJMS-UMDNJ, United States
Wilson S Tsai, Department of Heart, Lung and Esophageal Surgery, University of Pittsburgh Medical Center, United States
Supported by Pathology Department RWJMS-UMDNJ, United States
Correspondence to: Paula Andrea Rodriguez Urrego, Pathology Department, New Brunswick NJ 08901, One Robert Wood Johnson PL. MEB 212, United States. rodrigp2@umdnj.edu
Telephone: +1-908-7204531 Fax: +1-732-4188445
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Abstract

Extrarenal fibromuscular dysplasia causing gastrointestinal bleeding without other manifestations and especially sparing renal vasculature is uncommon. The diagnosis of this entity is usually made by radiographic appearance and the treatment is controversial. To our knowledge only seven cases of visceral fibromuscular dysplasia as a primary manifestation of the disease have been described, symptoms range from abdominal pain to gangrene. This is the first case of visceral fibromuscular dysplasia presenting with otherwise asymptomatic gastrointestinal bleeding, without bowel necrosis or ischemic changes. We provide a review of the literature.

Key words: Fibromuscular dysplasia; Extrarenal; Visceral; Gastrointestinal bleeding; Intimal fibroplasia

Rodriguez Urrego PA, Flanagan M, Tsai WS, Rezac C, Barnard N. Massive gastrointestinal bleeding: An unusual case of asymptomatic extrarenal, visceral, fibromuscular dysplasia. *World J Gastroenterol* 2007; 13(43): 5771-5774

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INTRODUCTION

Fibromuscular dysplasia (FMD) of extrarenal location, visceral or splanchnic, accounts for a small percentage of cases. Clinical presentation is variable, generally as occlusive or aneurysmal disease, and sometimes associated with a family history of FMD. Treatment is not standardized due to rarity of the entity and includes either segmental resection, with or without angioplasty or medical treatment with anticoagulation and thrombolysis. We present a case report and review of the literature.

CASE REPORT

A 38-year-old man was transferred to our facility with intractable gastrointestinal bleeding. On presentation at the referring institution, he denied shortness of breath, dizziness, lethargy or abdominal pain. Upper esophagogastroduodenosc opy performed at the referring institution, excluded an upper gastrointestinal source of bleeding. An angiogram suggested diffuse severe vasculitis involving celiac trunk, superior mesenteric artery and inferior mesenteric artery.

The patient's medical history was significant for multiple episodes of gastrointestinal bleeding and gastric ulcers. Surgical history was significant for small bowel resection, 6 mo prior to the present admission, where an exposed vessel was identified as the source of bleeding. A pathological diagnosis of angiodysplasia/vasculitis was made. Family history was unremarkable. The patient was a smoker and a social drinker but denied use of illicit drugs.

On admission his physical examination was positive for pallor and compensatory tachycardia with a stable blood pressure. Hemoglobin level was 8.4 g/dL despite multiple transfusions.

The patient was started on steroid therapy due to a history of vasculitis. However, antinuclear antibody titer (ANA) of < 1:16, excluded this possibility. Bleeding scan suggested a bleeding site in the small bowel bleeding most likely in the ileum. On the second day of admission an angiogram was performed with intent of embolization, and showed normal aorta and renal arteries and extensive vascular abnormalities involving all branches of the superior mesenteric artery, which included ectasia, bleading and narrowing; and marked ectasia of the inferior mesenteric artery and areas of hypervascularity. There was no evidence of contrast extravasation. Due to drop of hemoglobin and failure of medical treatment including attempted embolization, the patient was taken to surgery. Intraoperative colonoscopy showed obvious bleeding at the anastomosis site from the previous small bowel resection, in the ileum, with old blood distally. A segment of distal ileum, 6 cm away from the ileocecal valve was resected. No obvious bleeding site was identified.

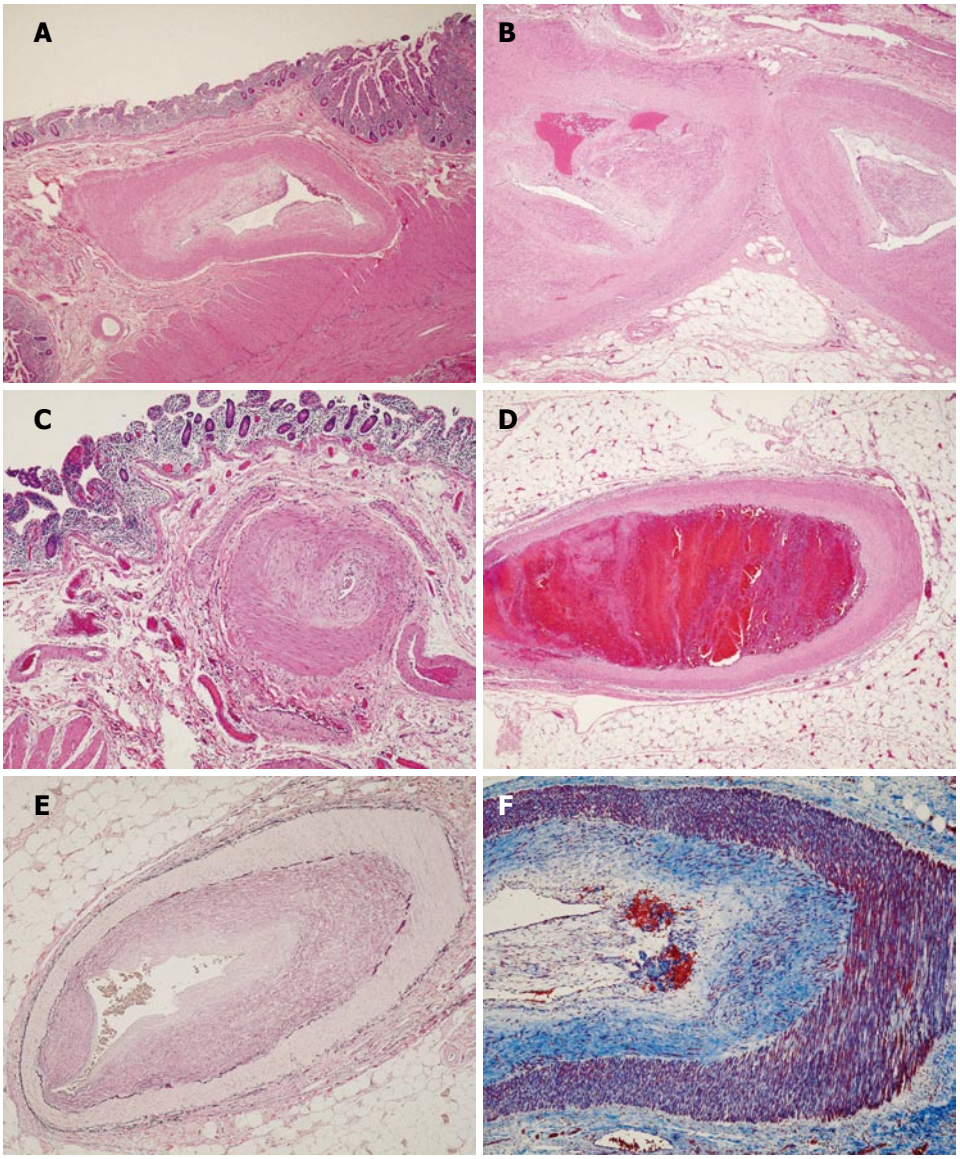

Figure 1 Microscopic sections showing marked thickening (**A**) and hyalinization (**B**) of medium sized vessel walls with prominent eccentric intimal proliferation with no arteritis, necrosis, inflammation and calcification (hematoxylin-eosin, original magnification × 200); sparing of small sized vessels (**C**) (hematoxylin-eosin, original magnification × 200); focal evidence of recent vascular thrombosis with early organization in one vessel (**D**) (hematoxylin-eosin, original magnification × 200); Verhoeff's Van Gieson elastic stain highlighting the prominent intimal hyperplasia (**E**) (original magnification × 200; Masson's trichrome stain highlighting a loose matrix of fibrous tissue replacing and expanding the intima (**F**) (original magnification × 200).

The specimen received consisted of a 37.5 cm segment of small bowel designated as "distal ileum" and a grossly unremarkable vermiform appendix. On opening the small bowel, the mucosal surface was intact without evidence of ulcers or masses. There were multiple yellow, tan slightly elevated areas of the mucosa, giving the appearance of papules, ranging from 0.3 to 0.5 cm. The previous anastomosis site was intact. Sections through the mucosa, perineal fat and mesentery showed thickened and tortuous submucosal, subserosal and mesenteric vessels, some of them contained clotted blood.

Microscopic sections (Figure 1) revealed marked thickening and hyalinization of medium sized vessel walls, with prominent eccentric intimal proliferation (Figure 1A and B) and apparent sparing of small sized vessels (Figure 1C). Focal evidence of recent vascular thrombosis with early organization was noted in one vessel (Figure 1D). Arteritis, necrosis, inflammation and calcification were absent. Verhoeff's Van Gieson elastic stain (Figure 1E) highlighted the prominent intimal hyperplasia. Masson's trichrome stain (Figure 1F) highlighted a loose matrix of fibrous tissue replacing and expanding the intima.

DISCUSSION

FMD was first described more than 68 years ago by Leadbetter, as a cause of hypertension, but was not until 1938 that it received an accurate pathological description by Mc Cormack, who reported four cases as "fibromuscular hyperplasia"[1,3]. Currently, FMD is well recognized as a nonatherosclerotic, noninflammatory vascular disease that most commonly affects young females, involving the renal and internal carotid arteries but has been described in almost all arterial beds in the body[1-9]. This entity accounts for less than 10% of cases of renal-artery stenosis[2]. Among 1200 cases of FMD studied by Meetinger[3], 60% involved renal arteries, 34% extracranial carotid and vertebral arteries, and less than 2.5% involved iliac, celiac and mesenteric arteries.

Two histopathological classifications of renal FMD have been proposed. In 1971, Harrison and McCormack[10] made the first classification, based on the arterial layer in which the lesion predominates: intimal fibroplasias, medial fibroplasias and adventitial fibroplasias. Intimal fibroplasia was found in less than 10% of patients with FMD, and can be confused with arteritis by angiography because it may appear as a focal, concentric stenosis or a long smooth

narrowing. Medial fibroplasia, characterized on imaging studies by a "string of beads appearance", represents the most common dysplastic lesion, with medial compromise and sparing of intimal and adventitial layers. Medial fibroplasias include two uncommon subtypes: perimedial fibroplasia and medial hyperplasia. Perimedial fibroplasia is characterized by a collar of elastic tissue at the junction of the media with the adventitia. Medial hyperplasia, corresponds to less than 1% of causes of renal stenosis, and consists of medial hyperplasia without loose matrix of fibrous tissue formation. The third category, also the rarest type of FMD is adventitial (periarterial) hyperplasia.

In 1975, Stanley[4], subdivided FMD into four categories, instead of three, according to histopathological findings: intimal fibroplasias, medial hyperplasia, medial fibroplasia and perimedial dysplasia. The first category, intimal fibroplasia accounts for 5% of cases of FMD appearing as irregular tubular stenosis in young patients and smooth focal stenosis in older persons, characterized histologically by an accumulation of irregularly arranged subendothelial mesenchymal cells within a loose matrix of fibrous connective tissue, often eccentric, with absence of inflammatory cells or foamy macrophages. The second, medial hyperplasia is characterized by increase in medial muscle, without demonstrable fibrotic changes, and accounts for less than 1% of cases. This category is difficult to differentiate roentographically from intimal fibroplasia. The third and most common category, medial fibroplasia, represents 85% of cases of renal FMD, and is most commonly seen in the pediatric population. It is characterized histologically by compromise of the outer media, or diffuse involvement of the entire media. Involvement of the outer media presents with compact fibrous connective tissue replacing smooth muscle peripherally, and a moderate accumulation of collagen in the inner media, separating disorganized smooth muscle. On the other hand, diffuse medial fibroplasia is characterized by severe disorganization, with disruption and replacement of smooth muscle by haphazard arrangements of fibroblasts and collagen. The last category, perimedial dysplasia, found in 10% of cases of FMD, is characterized by relatively acellular tissue in the region of the external elastic lamina that can be mistaken for dense collagen.

Both authors reported that macroaneurysms and dissections are the common complications of FMD. Aneurysms can present in any subtype, and their presence should not lead to a different diagnosis. To date, classifications are used in the literature, indistinguishably.

The etiology of FMD is unknown, but several factors, genetic, mechanical and hormonal, have been implicated[1-4,7-8]. Hormonal influence is considered due to the prevalence of the disease in hormonally active females. Although no association has been found between FMD and history of use of oral contraceptives or abnormalities of endogenous sex hormones, physiologic preconditioning of cellular elements to fibroblastic activities by estrogen has been shown. Mechanical factors, such as artery traction due to renal ptosis, could explain the increased frequency in the right kidney, compared to the left kidney. The stress involved in the stretch traction may predispose to fibroplasia directly by altering vessel wall tissues or indirectly by disrupting the vasa vasorum. Vasa vasorum of muscular arteries originates from branching of the parent vessel and arteries such as renal, extracranial carotid and external iliac arteries may have a normal paucity of vasa vasorum, making them prone to alteration. Genetic factors may be contributory, since FMD is more common among first degree relatives with renal FMD and among persons with angiotensin-converting-enzyme allele I (ACE-I).

The differential diagnosis is broad, but usually straight forward, once common etiologies are excluded[1,13]. Atherosclerosis typically occurs in older patients with cardiovascular risk factors and usually involves the origin or the proximal portion of the artery. In contrast, renal FMD occurs in younger patients, with few or no cardiovascular risks and frequently involves the middle or distal segments of artery. FMD is distinguished from active vasculitis, based on the absence of inflammation, anemia, thrombocytopenia or acute phase reactants. If histologic diagnosis is unavailable, angiography is unable to differentiate FMD and vasculitis, due to the extreme similarity in radiographic appearance, especially in the case of intimal fibroplasias, where both entities have identical manifestations, as illustrated in this case. Diseases affecting medium size vessels may induce identical histopathological changes in the vessel wall and should be considered in the differential diagnosis, including familial diseases such as neurofibromatosis and Friedreich's ataxia, endocrine diseases such as diabetes and homocystinuria, autoimmune diseases including any type of vasculitis, allograft rejection, infectious vasculitis such as Rickettsia, Rocky Mountain spotted fever, epidemic typhus, scrub typhus, Q-fever, pseudomonas, syphilis, fungi, plague, Whipple's disease, leptospirosis and schistosomiasis, toxic agents such as cocaine, coagulopathies such as thrombotic thrombocytopenic purpura, hemolytic uremic syndrome and disseminated intravascular coagulation, systemic hypertension, neoplastic entities such as intravascular lymphomatosis, endovascular papillary angioendothelioma or intravascular papillary endothelial hyperplasia, amyloidosis or emboli.

Visceral or splanchnic FMD accounts for 2.5% of cases of FMD and its clinical presentation has a wide spectrum[3,5-9,11-13]. When symptomatic, its presentation can be as occlusive disease or as aneurysmal disease. Occlusive disease ranges in spectrum from abdominal angina to intestinal gangrene. Of the seven case reports found in the literature with visceral FMD, two[8,9] had a family history of FMD or associated renal FMD. The other four[5,6,11,13] presented with only visceral involvement. The age ranged from 5 to 78 years. Three cases occurred in women and four in men. The initial presentation was acute abdomen in all the patients, with compromise of superior mesenteric artery in four patients, superior rectal artery in one, jejunal and sigmoid artery and colonic artery in one, and unspecified in the other patient. Two out of the seven patients presented with gastrointestinal bleeding. Histological analysis revealed mucosa with ischemic changes and FMD of different categories, intimal fibroplasia in three patients, medial fibroplasia in two patients, perimedial dysplasia in one patient and adventitial fibroplasia in one patient. Three patients died and four

of the seven survived. Among the successfully treated patients, three had surgery with a segmental resection, one of them combined with angioplasty. One patient was medically treated with anticoagulation and thrombolysis, without requiring any invasive procedure.

Interestingly enough, a study that originated from a case of ischemic proctitis, analyzed 50 rectums and their blood supply in patients with a median age of 61 years[11] showed that adventitial FMD was found in more than 50% of unselected, examined specimens, indicating that a relatively mild, asymptomatic form of adventitial FMD is common in later life.

Treatment for visceral FMD is more anecdotic and based on retrospective case series[1]. Revascularization through angioplasty without stent, segmental resections, anticoagulation and fibrinolysis has been used.

We present a case of extrarenal, visceral FMD, with intimal fibroplasia histopathologic type, causing intractable and recurrent gastrointestinal bleeding, without ischemia or necrosis of the bowel, who was successfully treated surgically with partial jejunectomy. The patient did well, and a year after small bowel resection he developed a ventral hernia, requiring laparoscopic and open repair. The specimen showed a large artery with fibromuscular and intimal hyperplasia. No new episodes of gastrointestinal bleeding or gastrointestinal symptoms have been documented.

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CASE REPORT

Well to moderately differentiated HCC manifesting hyperattenuation on both CT during arteriography and arterial portography

Soo Ryang Kim, Susumu Imoto, Hirotsugu Ikawa, Kenji Ando, Keiji Mita, Shuichi Fuki, Michiie Sakamoto, Yoshihiro Kanbara, Toshiyuki Matsuoka, Masatoshi Kudo, Yoshitake Hayashi

Soo Ryang Kim, Susumu Imoto, Hirotsugu Ikawa, Kenji Ando, Keiji Mita, Shuichi Fuki, Department of Gastroenterology, Kobe Asahi Hospital, Kobe 653-0801, Japan
Michiie Sakamoto, Department of Pathology, Keio University School of Medicine, Tokyo 160-8582, Japan
Yoshihiro Kanbara, Department of Surgery, Hyogo Cancer Center, Akashi 673-0021, Japan
Toshiyuki Matsuoka, Department of Radiology, Osaka City University Medical School, Osaka 545-8586, Japan
Masatoshi Kudo, Department of Gastroenterology, Kinki University School of Medicine, Osaka-Sayama 589-8511, Japan
Yoshitake Hayashi, Division of Molecular Medicine & Medical Genetics, International Center for Medical Research and Treatment, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
Correspondence to: Soo Ryang Kim, MD, Department of Gastroenterology, Kobe Asahi Hospital, 3-5-25 Bououji-cho, Nagata-ku, Kobe 653-0801, Japan. info@kobe-asahi-hp.com
Telephone: +81-78-6125151 Fax: +81-78-6125152
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Abstract

We present a rare case of well- to moderately-differentiated hepatocellular carcinoma (HCC) in a 71-year-old woman with hepatitis C virus-related cirrhosis and unusual radiologic features. A 20-mm hypoechoic nodule disclosed by ultrasound in segment two showed hyperattenuation on both computed tomography hepatic arteriography and computed tomography during arterial portography. Contrast-enhanced ultrasound revealed hypervascularity in the early vascular phase and defect in the post-vascular phase, with the same pattern detected by the two imaging techniques. SPIO-MRI revealed a hyperintense nodule. These findings were compatible with those of moderately-differentiated HCC. An ultrasound-guided biopsy showed histological features of well- to moderately-differentiated HCC characterized by more than two-fold the cellularity of the non-tumorous area, fatty change, clear cell change and mild cell atypia with a thin to mid-trabecular pattern. Further studies may provide insights into the correlation between tumor neovascularity in multistep hepatocarcinogenesis and dual hemodynamics, including the artery and the portal vein.

Key words: Hepatocarcinogenesis; CT hepatic arteriography; CT during arterial portography; Hyperattenuation; Dual hemodynamics; Well- to moderately-differentiated hepatocellular carcinoma

Kim SR, Imoto S, Ikawa H, Ando K, Mita K, Fuki S, Sakamoto M, Kanbara Y, Matsuoka T, Kudo M, Hayashi Y. Well to moderately differentiated HCC manifesting hyperattenuation on both CT during arteriography and arterial portography. *World J Gastroenterol* 2007; 13(43): 5775-5778

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INTRODUCTION

Recent advances in imaging have enabled clinicians to identify not only advanced HCC but also dysplastic nodules or early hepatocellular carcinoma (HCC). Moreover, clinicians can now obtain images by CT hepatic arteriography (CTA) and CT during arterial portography (CTAP) concurrently, and can evaluate the hemodynamics of lesions preoperatively[1,2].

To determine the treatment of choice for HCC, examinations by both CTA and CTAP are indispensable because of the high sensitivity of CTAP in detecting hepatic lesions and the capability of CTA of characterizing them. Moderately-differentiated HCC is generally demonstrated as a perfusion defect on CTAP and as an enhanced area on CTA, principally because of the reciprocal blood flow of these two features. We investigated a case of well- to moderately-differentiated HCC, manifesting hyperattenuation on both CTA and CTAP.

CASE REPORT

A 71-year-old woman with hepatitis C virus (HCV)-related cirrhosis was admitted to Kobe Asahi Hospital in January 2007 for further examination of a 20-mm hypoechoic nodule in segment two (S2). She had no history of alcohol, blood transfusion, or drug abuse. Six months earlier, the patient had undergone to radiofrequency ablation of a 20-mm totally necrotic HCC in segment 7 (S7).

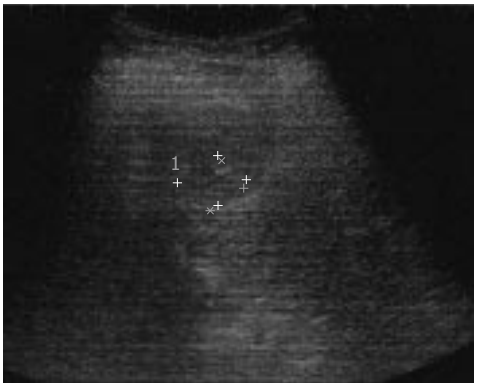

Figure 1 Ultrasound (US) image of a 20-mm hypoechoic nodule in segment 2 (S2).

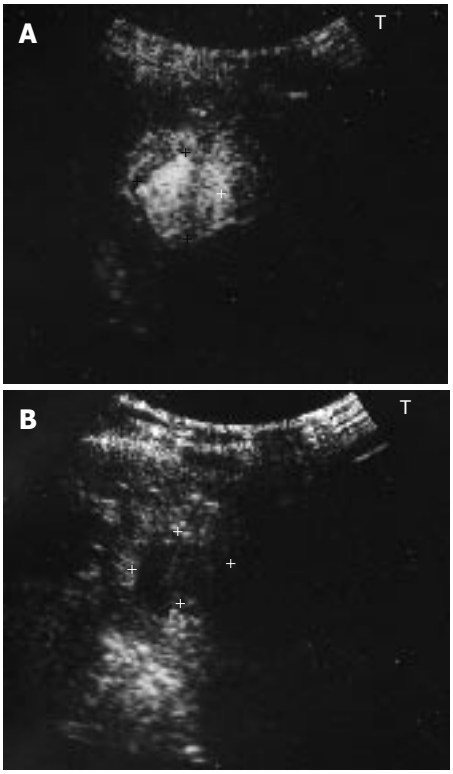

Figure 2 Contrast enhanced US of the nodule in S2. **A**: Hypervascularity in the early vascular phase; **B**: Defect in the post-vascular phase.

On admission, a physical examination showed no remarkable abnormalities. Serum HCV RNA was positive, and the HCV genotype was 1b. Serum hepatitis B virus (HBV) was negative for surface antigen, surface antibody, core antibody, and deoxyribonucleic acid (DNA). Laboratory studies disclosed the following abnormal values: platelets $6.0 \times 10^4/\mu L$ (normal 13.4-34.9), aspartate aminotransferase 50 U/L (10-40), alanine aminotransferase 46 U/L (5-40), alkaline phosphokinase 744 U/L (115-359), thymol turbidity 13.9 U (< 4.0), zinc surface turbidity 25.2 U (2.0-12.0), ICG R15 43% (0-10), γ globulin 52.8% (10.5-20.3). The levels of tumor markers were as follows: alpha-fetoprotein (AFP) 63.8 ng/mL (< 10.0), lens culinaris agglutinin A reactive fraction of alpha fetoprotein (AFP-L3) 4.6% (< 10.0), and protein-induced vitamin K absence (PIVKA II) 91 mAU/mL (<40).

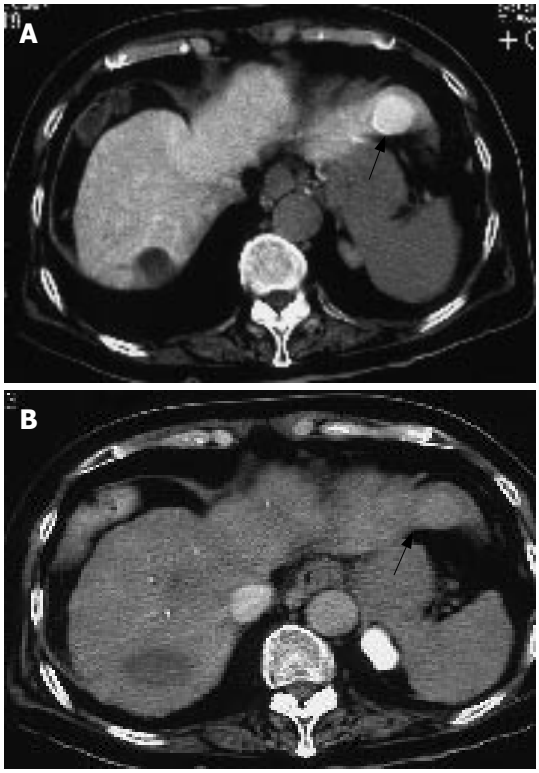

Figure 3 **A**: Hyperattenuation in S2 on computed tomography during arteriography (CTA); **B**: Hyperattenuation in S2 on computed tomography during arterial portography (CTAP).

Ultrasound (US) disclosed a 20-mm hypoechoic nodule in S2 (Figure 1). Contrast-enhanced US revealed hypervascularity in the early vascular phase (Figure 2A), and defect in the post-vascular phase (Figure 2B). Magnetic resonance imaging (MRI) revealed a high intensity nodule at both T1- and T2-weighted sequences. Contrast-enhanced MRI revealed a hypervascular nodule in the early phase and washout in the late phase. SPIO-MRI revealed a hyperintense nodule. Contrast-enhanced CT revealed an enhanced nodule in the early phase and washout in the late phase. Both CTA and CTAP revealed hyperattenuation (Figure 3A and B). Histologically, the nodule was well- to moderately-differentiated HCC characterized by more than two-fold the cellularity of the non-tumorous area, fatty change, clear cell change and mild cell atypia with a thin to mid-trabecular pattern (Figure 4A).

Immunohistochemical staining of heat shock protein (HSP) 70 (Figure 4B) was partly positive, and that of cyclase-associated protein (CAP) 2 was strongly positive (Figure 4C). Immunohistochemical staining of CD34 in HCC was positive in the sinusoidal blood space (Figure 4D). Immunohistochemically, CD68 was significantly underexpressed in the sinusoidal blood space compared with its expression in the non-HCC area (Figure 4E).

DISCUSSION

According to the classification by the International Working Party of the World Congress of Gastroenterology[3], hepatic nodules observed in patients with chronic liver disease can be subdivided into regenerative nodules

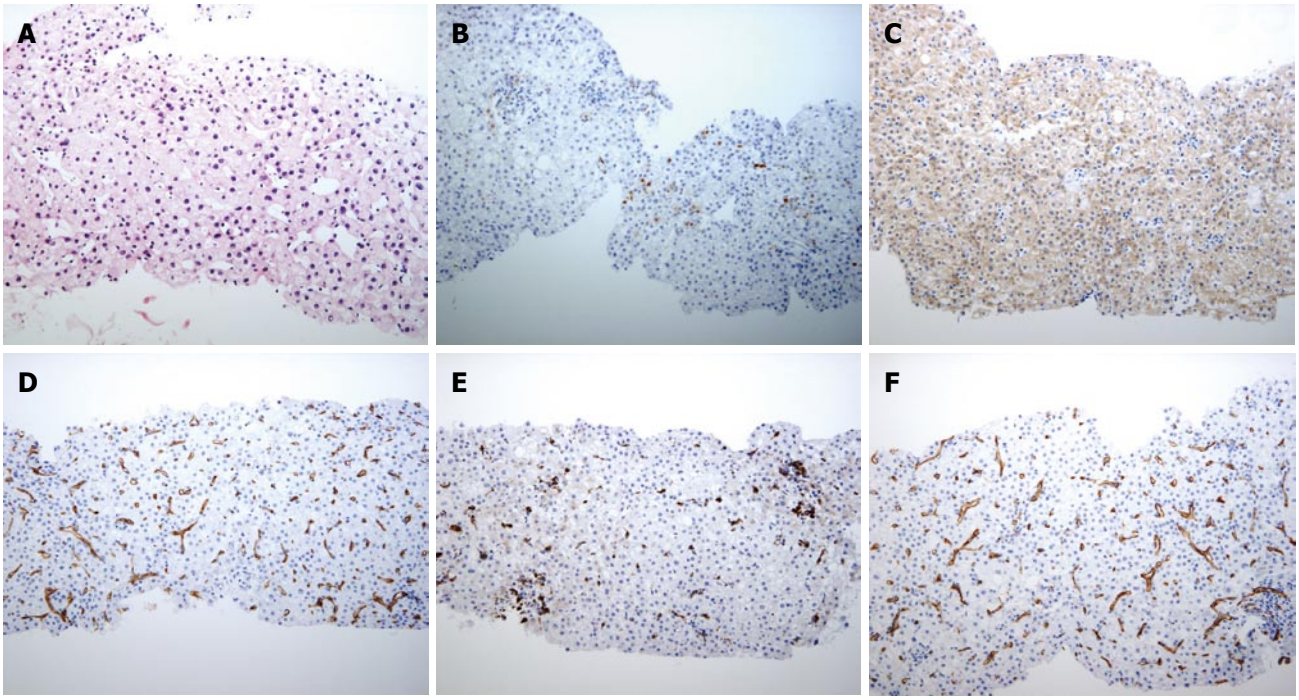

Figure 4 Histological features of a US-guided biopsy of a hyperechoic nodule in S2. **A**: Well- to moderately-differentiated HCC characterized by more than two-fold the cellularity of the non-tumorous area, fatty change, clear cell change and mild cell atypia with a thin to mid-trabecular pattern (HE×100); **B**: Immunohistochemical finding, HSP70 partly positive HCC cells; **C**: Immunohistochemical finding, CAP2 strongly positive HCC cells; **D**: Immunohistochemical staining of CD34 in the sinusoidal blood space is positive, showing capillarization; **E**: Immunohistochemical staining of CD68 Kupffer cells in the sinusoidal blood space is relatively less positive; **F**: Non-HCC area of immunohistochemical staining of CD68 Kupffer cells in the sinusoidal blood space.

(monoacinar and multiacinar), low-grade dysplastic nodules (LGDS), high-grade dysplastic nodules (HGDS), well-differentiated HCC (wHCC), moderately-differentiated HCC (mHCC), and poorly-differentiated HCC (pHCC), in the ascending order of histologic grades, representing a sequence of multistep hepatocarcinogenesis.

Several studies have shown sequential changes of hemodynamics in such hepatocellular neoplastic nodules and have reached similar conclusions: in the multistep developmental process of HCC, first, intratumoral hepatic arterial flow transiently decreases in the early stages of hepatocarcinogenesis and corresponds, histopathologically, to the obliteration of pre-existing hepatic arteries[2]; next intratumoral portal flow decreases gradually with the elevation of the histopathologic grades of hepatocellular nodules; and then hepatic arterial flow shows a gradual increase[4].

As is well documented through imaging techniques, such as CTA and CTAP, and histopathologically, the hepatocarcinogenetic process involves a gradual decrease in portal blood flow within the lesion and a reciprocal increase in hepatic arterial blood.

Although the HCC lesion is usually demonstrated as an iso- or hypoattenuated mass on CTAP, hyperattenuated lesions on CTAP are rare and limited to observations made in Japan[5-7].

Hyperattenuation on CTAP has been postulated as being reciprocal compensation for portal venous flow and hepatic arterial flow[5]; however, it is difficult to explain such hyperattenuation on CTAP by this premise because of the crucial gradual pressure between the hepatic artery and the portal vein. Although this hypothesis has not been pathophysiologically proven yet, one possible explanation[8] could be the following.

Abnormal thick-walled tumor vessels or tumor neovascularity may not necessarily carry arterial blood, considering the unique dual blood supply system of the originating organ, the liver. Tumor vessels may be communicating predominantly with the portal venous system through pre-existing intratumoral portal veins at the early stage of hepatocarcinogenesis (LGDN to wHCC), when both pre-existing arteries and portal veins decrease in number and when tumor angiogenesis may already have begun.

Net portal perfusion seen on CTAP in such tumors may reflect the sum or an additional effect of the reduced preexisting portal vein and the increased tumor vessels. These two factors may thus cancel out each other, resulting in isoperfusion on CTAP. When tumor angiogenesis exceeds the loss of a preexisting portal vein, the lesion may exhibit hyperattenuation on CTAP. Despite the communication between tumor vessels and portal veins, the tumor may become hypoxic because of the paucity of oxygen in portal venous blood compared with arterial blood. The hypoxia may thus induce gradual angiogenesis. As the histologic grades of the liver nodule advance, tumor vessels increase and may start to communicate with the arterial system as well, either through preexisting arteries or directly through outside arteries. Portal communication of the tumor vessels may gradually decrease; instead, arterial communication becomes gradually dominant. In mHCC and pHCC, therefore, the tumor may be fed exclusively by

arterial supply.

We have previously presented a rare case of well-differentiated HCC manifesting hypoattenuation on CTA and hyperattenuation on CTAP[7]. Takayasu et al[6] also have presented three such cases of HCC. These observations give rise to one possible explanation of the present case manifesting hyperattenuation on both CTA and CTAP: in the course of dedifferentiation from well- to moderately-differentiated HCC, there may be an intermediate phase between the manifestation of hypoattenuation on CTA and hyperattenuation on CTAP and the manifestation of hyperattenuation on CTA and hypoattenuation on CTAP. To the best of our knowledge, this is the first case of well- to moderately-differentiated HCC manifesting hyperattenuation on both CTA and CTAP.

Histopathologically, the tumor showed well- to moderately-differentiated HCC characterized by more than two-fold the cellularity of the non-tumorous area, fatty change, clear cell change and mild cell atypia with a thin to mid-trabecular pattern. Varying degrees of fatty changes may be one of the significant morphological markers of malignant transformation in the nodule, as observed in our US guided biopsy specimen.

Immunohistochemically, HSP70 is significantly overexpressed in early HCC, compared with its expression in dysplastic nodules, reaching 80% in most cases of well-differentiated HCC[9].

All cases of dysplastic nodules have been negative or focally positive (5%-10% of the lesions) for CAP2; in contrast, most cases of HCC (27 of 29 cases) have been partly positive for CAP2. Of the lesions, 70%-100% have been positive in the advanced components, and the positivity of well-differentiated HCC has ranged from 10% to 100%[10].

Positive immunohistochemical staining of both HSP70 and CAP2 confirmed the diagnosis of well- to moderately-differentiated HCC in the present case.

Positive immunohistochemical staining of CD34 in endothelial cells in the sinusoidal blood space has shown capillarization[11]. The immunohistochemical staining of CD34 was compatible with the hypervascularity of our tumor, as shown by imaging studies. Relatively less positive staining of CD68 of Kupffer cells in the sinusoidal blood space revealed the dedifferentiation. This finding was compatible with the imaging studies based on SPIO-MRI.

Further studies on sequential homodynamic change in hepatocarcinogenesis are indicated.

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CASE REPORT

Minute signet ring cell carcinoma occurring in gastric hyperplastic polyp

Shoji Hirasaki, Seiyuu Suzuki, Hiromitsu Kanzaki, Kohei Fujita, Shuji Matsumura, Eiji Matsumoto

Shoji Hirasaki, Seiyuu Suzuki, Hiromitsu Kanzaki, Kohei Fujita, Shuji Matsumura, Eiji Matsumoto, Department of Internal Medicine, Sumitomo Besshi Hospital, 3-1 Ohji-cho, Niihama 792-8543, Japan
Correspondence to: Dr. Shoji Hirasaki, Second Department of Internal Medicine, Sumitomo Besshi Hospital, 3-1 Ohji-cho, Niihama 792-8543, Japan. shoji_hirasaki@ni.sbh.gr.jp
Telephone: +81-897-377111 Fax: +81-897-377121
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Abstract

We describe a 45-year-old woman with minute signet ring cell carcinoma occurring in a gastric hyperplastic polyp. A biopsy specimen obtained from the gastric hyperplastic polyp revealed signet ring cell carcinoma. Endoscopic mucosal resection (EMR) was performed to confirm the diagnosis. Histological examination of the EMR specimen revealed focal signet ring cell carcinoma in the hyperplastic polyp. There are few cases of gastric hyperplastic polyp associated with signet ring cell carcinoma.

Key words: Minute gastric cancer; Signet ring cell; Endoscopic mucosal resection

Hirasaki S, Suzuki S, Kanzaki H, Fujita K, Matsumura S, Matsumoto E. Minute signet ring cell carcinoma occurring in gastric hyperplastic polyp. *World J Gastroenterol* 2007; 13(43): 5779-5780

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INTRODUCTION

Patients with gastric hyperplastic polyps may present with anemia, abdominal pain or gastric outlet obstruction[1,2]; therefore, most endoscopists agree that large gastric polyps or polyps associated with complications should be removed endoscopically or surgically. On the other hand, signet ring cell carcinoma rarely occurs in gastric hyperplastic polyps; however, no standardized therapy for such cases has been established. Herein, we report a rare case of a Japanese woman diagnosed with minute signet ring cell carcinoma in a gastric hyperplastic polyp and treated with endoscopic mucosal resection (EMR).

CASE REPORT

A 45-year-old woman visited our hospital for a follow-up study of a gastric polyp. She had been diagnosed with gastric hyperplastic polyp on the posterior wall of the middle third area 5 years previously. Her body temperature was 36.4℃, blood pressure was 126/78 mmHg, and radial pulse rate was 66 beats/min and regular. She had neither anemia nor jaundice. A neurological examination revealed no abnormal findings and there was no lymphadenopathy. No specific family history was identified. Routine hematological examination and biochemical tests were within normal limits. Serum anti-*H pylori* immunoglobulin G (IgG) antibody was positive. Endoscopic examination of the upper digestive tract revealed a small gastric hyperplastic polyp in the posterior wall of the gastric body (Fig. 1). The first biopsy specimen obtained from the polyp showed signet ring cell carcinoma. However, the biopsy specimen obtained repeatedly (three times) from the lesion revealed hyperplastic foveolar epithelium. Thus, the definite diagnosis could not be made. The patient underwent an EMR for histological confirmation. The protruding lesion, 6 × 5 mm in size, was resected completely with a safe lateral and vertical margin (Fig. 2). Histological examination showed neoplastic cells with signet ring features surrounded by the tissue of the hyperplastic polyp (Fig. 2). The protruding lesion was diagnosed as minute signet ring cell carcinoma in a hyperplastic polyp with mucosal invasion, ly0, and v0.

DISCUSSION

This case involved the unusual association of a gastric hyperplastic polyp and focal signet ring cell carcinoma. Histological features in this case fulfilled the criteria of Nakamura *et al*[3] for the malignant transformation of hyperplastic polyps: (1) coexistence of benign and malignant parts in the same polyp; (2) existence of sufficient evidence that the benign area had previously been a benign polyp; and existence of sufficient cellular and structural atypia in the malignant area to be diagnosed as cancer.

The relationship between gastric hyperplastic polyp and gastric cancer remains unknown. In a study of gastric polyps[2,4] we found that hyperplastic polyps are the most common; nearly 85%-91% of all polyps were hyperplastic polyps. In another report, the incidence of gastric hyperplastic polyps was reported to be 28.3% in one series of 5515 gastric polyps by Stolte *et al*[5]. As gastric

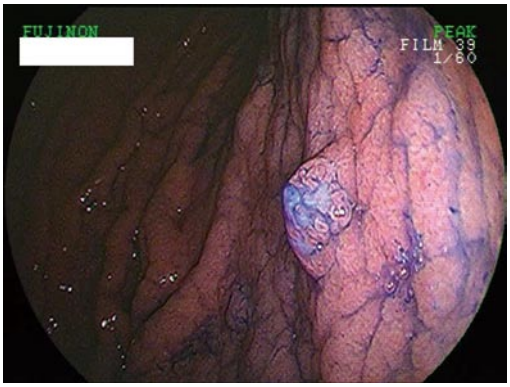

Figure 1 Endoscopic appearance of the gastric hyperplastic polyp in the posterior wall of the gastric body.

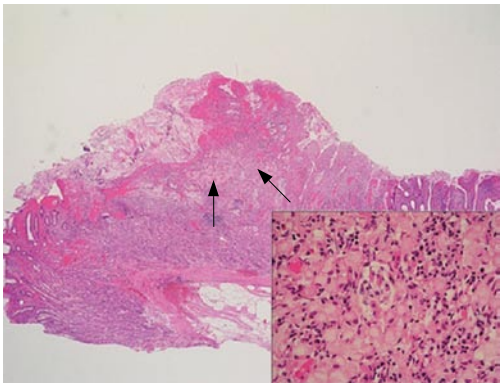

Figure 2 The resected specimen obtained by endoscopic mucosal resection showing localized neoplastic cells with signet ring features limited to the mucosal layer (arrows) (HE, × 10, × 100).

hyperplastic polyps are common, we should identify the relationship between gastric hyperplastic polyps and gastric cancer. It is generally acknowledged that the natural course of hyperplastic polyps does not include transformation to carcinoma, although hyperplastic polyps occasionally associate with gastric cancer[6-8]. In these reports, most hyperplastic polyps harboring cancer were larger than 1 cm in size[6-8]. The malignant transformation of a hyperplastic polyp is considered to relate to the size and macroscopic type; as the polyp grows larger and becomes semipedunculated or pedunculated, adenomatous or dysplastic foci appear first, followed by the cancerous lesion. Most adenocarcinomas found within hyperplastic polyps are the differentiated type. Few cases of signet ring cell carcinoma occurring in gastric hyperplastic polyps have been reported[4,9].

In the present case, the hyperplastic polyp, 6 mm in diameter, was associated with focal signet ring cell carcinoma and was diagnosed by endoscopic biopsy by chance. However, we could not locate the cancer in the polyp by careful observation with endoscopy. Thus, association of hyperplastic polyp and gastric cancer should generally be taken into consideration when endoscopists detect gastric hyperplastic polyps. Endoscopists should aggressively obtain biopsy specimens from hyperplastic polyps even if they are small.

In conclusion, we report the case of a woman diagnosed with minute signet ring cell carcinoma in a hyperplastic gastric polyp. This case emphasizes that small gastric hyperplastic polyp may be associated with gastric cancer, and periodic follow-up endoscopy and careful observation are necessary when treating patients with gastric hyperplastic polyp, even when it is less than 1 cm.

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CASE REPORT

Mucinous cyst exhibiting severe dysplasia in gastric heterotopic pancreas associated withe gastrointestinal stromal tumour

Antony Kaufman, David Storey, Cheok Soon Lee, Rajmohan Murali

Antony Kaufman, Cheok Soon Lee, Rajmohan Murali, Department of Anatomical Pathology, Sydney Cancer Centre, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia
David Storey, Department of Surgery, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia
Rajmohan Murali, Sydney Melanoma Unit, Sydney Cancer Centre, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia
Correspondence to: Dr. Rajmohan Murali, Department of Anatomical Pathology, Royal Prince Alfred Hospital, Missenden Road, Camperdown, NSW 2050, Sydney, Australia. rajmohan.murali@email.cs.nsw.gov.au
Telephone: +61-2-95157458 Fax: +61-2-95158405
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Abstract

Heterotopic pancreatic tissue within the stomach is rare and dysplasia within heterotopic pancreatic tissue is very rare. We present the first report of a patient with concurrent occurrence of heterotopic pancreas in the stomach with a gastrointestinal stromal tumour.

Key words: Dysplasia; Gastrointestinal stromal tumour; Heterotopic pancreas; Mucinous cyst; Stomach

Kaufman A, Storey D, Lee CS, Murali R. Mucinous cyst exhibiting severe dysplasia in gastric heterotopic pancreas associated with gastrointestinal stromal tumour. *World J Gastroenterol* 2007; 13(43): 5781-5782

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INTRODUCTION

Heterotopic pancreas is a relatively common lesion found in approximately 1 in 500 abdominal laparotomies[1] with approximately 62% found in the stomach[2]. Pancreatic heterotopia is defined as pancreatic tissue lacking vascular or anatomical continuity with the normal pancreas[1]. Symptoms associated with heterotopic pancreas are rare, but when present are usually associated with a gastric site. Symptoms may be due to mass effect such as pyloric obstruction, ulceration or bleeding, or due to pancreatic diseases such as pancreatitis, cyst formation or pancreatic neoplasia. Dysplasia and malignancy within pancreatic heterotopia are rare events[3]. The synchronous occurrence of gastrointestinal stromal tumour (GIST) and heterotopic pancreas has not, to our knowledge, been previously reported.

CASE REPORT

A 71 year-old female was admitted to our hospital for melaena (77 g/L haemoglobin). She underwent endoscopy, and two lesions were visualised in the gastric antrum, one with overlying mucosal ulceration. The lesions were not biopsied as they were scheduled for resection. On the following day, both lesions were resected. Macroscopically the lesions measured 70 mm × 50 mm × 40 mm and 15 mm × 15 mm × 12 mm. The former was a solid tumour with a fleshy tan cut surface, while the latter was a solid and cystic lesion containing a single cyst (10 mm in diameter) filled with mucinous material. The solid lesion with surface ulceration was a gastrointestinal stromal tumour (intermediate risk, uncertain malignant potential)[4] composed of fascicles of spindle cells exhibiting mild nuclear pleomorphism and up to 4 mitotic figures per 50 high power fields (Figure 1). Large areas of tumour necrosis were present. Surface ulceration was present, which was the likely source of melaena.

The smaller lesion was composed of pancreatic ducts and acini within the gastric submucosa and focally extended into the muscularis propria. Within this area was a cyst partly lined by mucinous epithelium while in some areas the lining epithelium was denuded. The mucinous epithelium exhibited moderate to severe dysplastic cytoarchitectural features (tufted and micropapillary architecture, along with nuclear enlargement, stratification, coarsely granular chromatin and nucleolar prominence) (Figure 2). Around the cyst there were small pools of extracellular mucin, associated with a chronic inflammatory response suggestive of partial rupture. Adjacent smaller dilated ducts were lined by non-dysplastic columnar mucinous epithelium. No evidence of stromal invasion was present. The cyst lining cells were positive for CK7 and CK20 (Figure 2), the latter was localized in the dysplastic areas. The intracytoplasmic mucin was positive for alcian blue pH 0.5 and periodic acid Schiff.

The gastric mucosa adjacent to the lesions showed active chronic gastritis, *H pylori* organisms and intestinal metaplasia.

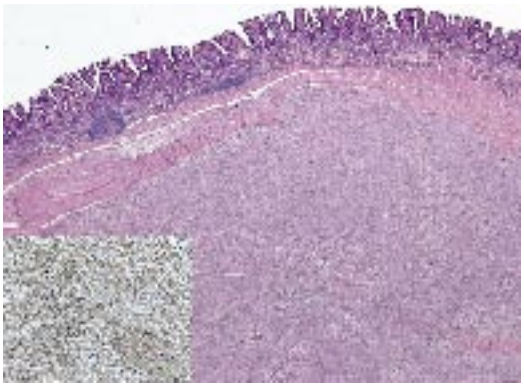

Figure 1 Gastrointestinal stromal tumour composed of fascicles of spindle cells. The tumour cells are positive for CD117 (inset).

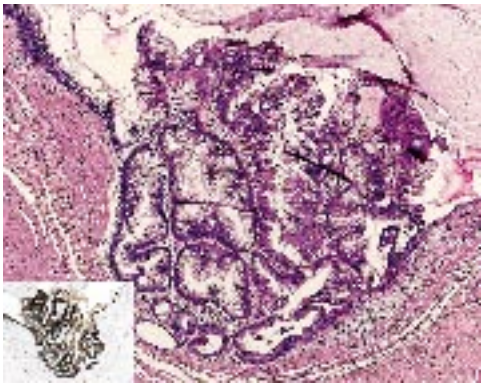

Figure 2 Severely dysplastic mucinous epithelium lining mucin-filled cyst in heterotopic pancreas. The dysplastic epithelium is positive for CK20 (inset).

DISCUSSION

Heterotopic pancreas is a relatively common lesion most often present in the gastric antrum seen macroscopically as a round or lobulated white to yellow which can be up to a few centimetres in dimension[2]. Diagnosis is difficult and often not made until surgical removal of the lesion. Heterotopic pancreas has been classified into three types by Heinrich: class I is typical pancreatic tissue with acini, ducts and islet cells, class Ⅱ shows a large number of acini and few ducts, and class Ⅲ shows numerous ducts with few acini or islet cells[5]. Neoplasms arising in heterotopic pancreatic tissue are rare[6] and include borderline mucinous cystic tumour[7], adenocarcinoma[6], mucinous cystadenocarcinoma[8], acinar cell carcinoma[2], islet cell tumour[9], or solid and papillary neoplasm[10]. Cystic degeneration without malignant change appears to be more common and may mimic mucinous carcinoma from another primary site[11]. The case report by Naqvi *et al*[7] includes a description of jejunal pancreatic heterotopia with cystically dilated ducts lined by mucinous epithelium showing low grade dysplastic cytoarchitectural features. They diagnosed a borderline mucinous cystic tumour without documenting the presence of ovarian type stroma. Of the reported cases of malignancy few have included reference to dysplastic or pre-malignant change[8,12]. These reports note the presence of dysplasia or carcinoma *in-situ* within the heterotopic pancreas adjacent to invasive ductal adenocarcinoma. Given the morphological appearances of the severe dysplasia seen in our patient, it is likely that the changes represent a pre-malignant change akin to that reported in orthotopic pancreas under the rubric "pancreatic intraepithelial neoplasia" (PanIN). Although malignant change within heterotopic pancreas is rare, we recommend that in the presence of dysplastic change within heterotopic pancreas tissue, the entire lesion should be sampled and examined histologically to exclude the presence of invasive malignancy.

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CASE REPORT

Association of liver cirrhosis related IgA nephropathy with portal hypertension

Georgios Kalambokis, Leonidas Christou, Dimitrios Stefanou, Evdokia Arkoumani, Epameinondas V Tsianos

Georgios Kalambokis, Leonidas Christou, Epameinondas V Tsianos, 1st Division of Internal Medicine and Hepato-gastroenterology Unit, University Hospital, Ioannina, Greece
Dimitrios Stefanou, Evdokia Arkoumani, Department of Pathology, University Hospital, Ioannina, Greece
Correspondence to: Dr. Epameinondas V Tsianos, Professor, Medical School of Ioannina, 45110 Ioannina, Greece. geodora@mail.gr
Telephone: +30-26510-97501 Fax: +30-26510-97016
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Abstract

A high incidence of IgA nephropathy has been reported in patients with liver cirrhosis, though, clinically evident nephrotic syndrome is very uncommon. Impaired hepatic clearance of circulating IgA immune complexes and subsequent deposition in renal glomeruli has been considered principally in the pathogenesis of liver cirrhosis associated IgA nephropathy. Here we report on a patient with cryptogenic liver cirrhosis and splenic vein thrombosis, who presented with nephrotic syndrome. Renal biopsy showed findings consistent with IgA nephropathy. Lower endoscopy showed features of portal hypertensive colopathy. Following initiation of propranolol and anticoagulant treatment to reduce portal pressure, a gradual decrease of proteinuria and hematuria to normal range was noted. The potential pathogenetic role of portal hypertension in the development of IgA nephropathy in cirrhotic patients is discussed.

Key words: IgA nephropathy; Nephrotic syndrome; Portal hypertension; Liver cirrhosis

Kalambokis G, Christou L, Stefanou D, Arkoumani E, Tsianos EV. Association of liver cirrhosis related IgA nephropathy with portal hypertension. *World J Gastroenterol* 2007; 13(43): 5783-5786

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INTRODUCTION

IgA nephropathy (IgAN) is a well known concomitant of liver cirrhosis (LC) with largely unknown pathogenesis[1,2]. Most of the literature has focused on the causative role of impaired clearance of circulating IgA immune complexes (IgAIC) by the diseased liver with subsequent intraglomerular deposition[1,3-5]. Other reports suggested that some etiological factors of chronic liver disease may be associated per se with development of IgAN[6-11]. We report the case of a patient with portal hypertension (PH) due to cryptogenic LC and splenic vein thrombosis (SVT) presented as nephrotic syndrome (NS), caused by IgAN. Proteinuria resolved after the introduction of propranolol and oral anticoagulation. The association of PH with IgAN is reviewed and potential pathogenetic mechanisms through which PH can cause IgAN in cirrhotic patients are proposed.

CASE REPORT

A 34-year-old man was evaluated for nephrotic range proteinuria and LC. Seven days before, he was admitted to another hospital with a 6-mo history of ankle swelling and periorbital oedema, and a 3-wk history of increased abdominal distention. Abdominal ultrasound showed a nodular liver, moderate ascites, and splenomegaly; the kidneys had normal dimensions and structure. Diagnostic aspiration of peritoneal fluid showed no evidence of infection; the serum ascites albumin gradient was consistent with PH (1.2). A large volume paracentesis was performed and daily treatment with furosemide 40 mg and spironolactone 100 mg was initiated.

On admission, the patient had mild peripheral oedema and ascites. Past history, including blood transfusion and alcohol consumption, was unremarkable, and he denied taking regular medication. There was no history of family renal or liver disease. Abnormal laboratory results were as follows: hemoglobin 114 g/L (normal range, 130-180), platelet count 79 000/mm^3 (130 000-400 000), international normalized ratio 1.2, fibrinogen 3.2 g/L (2-4), plasma D-dimers 1310 mg/L (< 200), serum urea 9.86 mmol/L (1.7-8.3), serum creatinine 88.4 μmol/L (53-106), total bilirubin 20.5 μmol/L (5.1-17), alanine aminotransferase 59 U/L (4-36), serum total protein 56 g/L (60-78), and serum albumin 22 g/L (32-45). Urinary protein was 3.6 g/day (< 0.06), red cell content 80-100 per field, white cell content 0-2 per field. Serum IgA was 7.1 g/L (0.9-3.2), IgG 15.7 g/L (8-15), and IgM 2.3 g/L (0.5-3). Serum C3 and C4 were 0.76 g/L (0.86-1.84) and 0.1 g/L (0.2-0.58), respectively. Chest radiography and echocardiography were normal. Stool examination for occult blood was positive with a ferritin of 5 ng/mL (6-80).

Liver biopsy confirmed LC but failed to disclose the aetiology of liver disease. The results of a thorough diagnostic work-up, including PCR for HBV and HCV, antimitochondrial antibodies, antinuclear antibodies, anti-smooth muscle antibodies, anti-liver-kidney microsomal antibodies, soluble liver antigen, alpha1-antitrypsin, copper and iron studies, and cryoglobulins, were also negative. Upper gastrointestinal endoscopy showed 1st degree esophageal varices and congested gastric mucosae. Colonoscopy revealed hyperemia, mucosal oedema, and friability throughout the entire colon, indicative of PHC. Abdominal computed tomography and venography revealed SVT and extensive venous collateral circulation; there was no evidence of splenorenal shunt, and portal or renal vein thrombosis. Investigation for acquired and inherited thrombophilic factors revealed the G20210A heterozygous mutation of prothombin. Other coagulation defects, including deficiencies of natural inhibitors of coagulation (protein C, protein S, antithrombin), factor V Leiden mutation, TT677 mutation of methylene-tetrahydrofolate reductase, and anticardiolipin antibodies, were not detected.

A renal biopsy demonstrated a diffuse endocapillary glomerulonephritis with a small proportion of crescends. The immunofluorescent study revealed IgA deposits of high intensity, localized within the mesangium of all glomeruli, as well as lesser amounts of IgM and C3. The electron-microscopic study showed electron-dense deposits under the basement membrane of capillaries that extended to the paramesangial region. A few intramembranous deposits were also seen (Figure 1).

Propranolol, increasing to 60 mg daily to achieve a reduction of heart rate by 25%, the vitamin K antagonist acenocoumarol, targeting an INR in the range of 2-3, and iron sulfate were added to diuretic treatment. Renal protein excretion decreased to normal range within 20 d together with a reduction of IgA levels (3.4 g/L) and disappearance of hematuria; ascites and oedema gradually resolved. One month later, repeated tomography showed patent splenic vein and a decrease in collateral circulation and size of spleen.

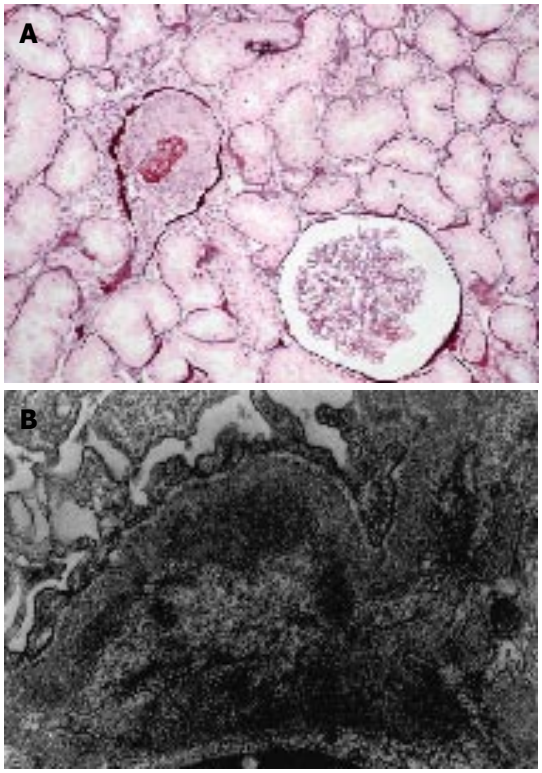

Figure 1 **A**: Light microscopy study showing endocapillary and extracapillary proliferation. A large cellular crescend is observed in the left upper glomerulus (silver methoxamine stain, × 200); **B**: Electron-microscopic study showing intramembranous and paramesangial electron-dense deposits (× 13 000).

DISCUSSION

IgAN associated with liver disease is the commonest form of secondary IgAN[12]. Glomerular deposits of IgA and fewer amounts of other immunoglobulins and C3 have been noted in 35% to 90% of cirrhotic patients[1,2]. The majority of IgA synthesis in human is mucosal, predominantly polymeric (pIgA) with two isotypes, IgA1 and IgA2, in about equal proportions. Normally, little mucosal pIgA reaches the circulation[12]. In patients with chronic liver disease, increased circulating IgA against gut flora and IgAIC have been reported[5,13]. Serum complement factor levels may be low due to activation of complement components by immune complexes, hepatic hyposynthesis, or both[14]. PIgA2 is mainly eliminated through the hepatic asialoglycoprotein receptor (ASGR) whereas only a small percentage of pIgA1 is cleared through this pathway[15], which explains the higher proportion of circulating IgA2 in chronic liver disease[16]. The majority of pIgA is then released back to the circulation in degraded forms[17]. Kupffer cells participate in the removal of IgA expressing specific Fc receptors[18]. PIgA1 is the predominant form in mesangial deposits[19], yet no antigens have been identified within the mesangial deposits.

LC-related IgAN is usually clinically silent[1]. Nakamoto *et al*[20] reported NS in 1.6% of a series of 752 cirrhotic patients. Microscopic but rarely gross hematuria may occur, but it is less common than in patients with primary IgAN[1,20].

The pathogenesis of LC-related IgAN remains uncertain. Impaired hepatic clearance of IgAIC due to reduced phagocytic[1,3-5] or ASGR[6] activity, which subsequently are deposited in the renal glomeruli, have been considered principally in the literature. However, there is no correlation between the degree of liver damage and the IgAIC levels[16]. In addition, a direct association of various causes of chronic liver disease, such as hepatitis viruses B[6] and C[7] infections, heavy alcohol consumption[8], autoimmune hepatitis[9], primary hemochromatosis[10], and alpha1-antitrypsin deficiency[11] with immunological renal injury has been suggested.

In the present case, IgAN was secondary to LC of unknown etiology complicated by PH and portosystemic shunting, as shown by the imaging results, and the presence of PHC[21] and esophageal varices. Proteinuria and hematuria resolved after introduction of propranolol to improve PHC[21] by decreasing portal pressure and tributary flow[22]. No significant renal effects of propranolol have been reported in cirrhotic patients to

justify decrease of proteinuria[23]. SVT has been associated with left-sided PH in non-cirrhotic subjects[24]. Therefore, acenocumarol may have exerted an additive portal hypotensive effect in our patient by restoring SVT, possibly related to PTHR A20210 mutation[25] or NS per se[26]. Renal vein thrombosis[26] or spontaneous splenorenal shunt[27], which might have been involved in the development and progress of IgAN in our patient, were not confirmed by computed tomography and venography.

Consistent with our observations, three previous case reports highlighted the pivotal role of PH in the development of LC-related IgAN[28-30]. Nakamura *et al*[28] reported a progressive fall in proteinuria together with clinical remission of NS after initiation of propranolol in a patient with cryptogenic LC. In another case of non-cirrhotic PH and IgAN induced NS, portacaval anastomosis decreased portal pressure causing proteinuria and hematuria to disappear[29]. Finally, Babbs *et al*[30] described a case of IgAN in a non-cirrhotic patient with a large splenic artery aneurysm causing PH and extensive collateral circulation. Splenectomy and aneurysm resection induced remission of the NS and hematuria.

The portosystemic shunting that results from PH may cause IgA to deviate from hepatic uptake and depolymerization[1]. Therefore, reduction of PH is expected to increase hepatic processing of IgAIC. PH also decreases small intestinal motility promoting bacterial overgrowth[31,32], while in the same time increases intestinal permeability[33]. In this regard, increased circulating IgA and IgAIC may represent an exaggerated response of immune system to excess gut antigen exposure[34,35], resulting from diminished mucosal integrity and spillage of IgA into the circulation. Another mechanism could involve the endotoxemia that frequently accompanies LC with PH[36], suggested to be generated by gut flora through bacterial translocation[37,38] favored by dysfunction of intestinal barrier[33,37]. Previous literature showed that endotoxemia may decrease hepatic ASGR binding[39]. Therefore, portal hypotensive treatment may decrease bacterial translocation and endotoxin levels[40], which could restore ASGR function.

In conclusion, PH seems to play a significant role in the pathogenesis of IgAN associated with LC. In this point of view, a decrease in PH is likely to reduce renal protein excretion in patients with significant proteinuria. Further studies are needed to assess if the severity of PH is related with the magnitude of renal IgA deposition.

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CASE REPORT

Cerebral metastasis from hepatoid adenocarcinoma of the stomach

Sheng Zhang, Mi Wang, Yi-Hui Xue, Yu-Peng Chen

Sheng Zhang, Mi Wang, Yi-Hui Xue, Yu-Peng Chen, Department of Pathology, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, Fujian Province, China
Correspondence to: Dr. Sheng Zhang, Department of Pathology, The First Affiliated Hospital of Fujian Medical University, Fuzhou 350005, Fujian Province, China. zhgshg@126.com
Telephone: +86-591-87982733 Fax: +86-591-83318716
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Abstract

We first report a rare case of metastasis from gastric hepatoid adenocarcinoma (HAC) to cerebral parenchyma, in a 50-year-old Chinese patient. He complained of a one-month history of a paroxysm of headache in the left temple and pars parietalis accompanied with binocular caligation caligo, insensible feeling of limbs and transient anepia. Magnetic resonance (MR) imaging revealed a spherical occupying lesion in the left posterior-temple lobe which was clinically diagnosed as a metastatic tumor. Three years ago, the patient accepted total gastrectomy as he was pathologically diagnosed at gastroscopy having an adenocarcinoma. Eight months after gastrectomy, the occupying lesion in liver was detected by ultrasound and CT, and he accepted transcatheter arterial embolization. Before operation of the brain metastasis, no obvious abnormality was found in liver by ultrasound. Histopathological characteristics of the brain tumor were identical to those of stomach tumor. The growth pattern of both tumors showed solid cell nests. The tumor cells were polygonal, and had abundant eosinophilic cytoplasm and round nuclei with obvious nucleoli. Sinusoid-like blood spaces were located between nodular tumor cells. Immunohistochemistry-stained tumor cells were positive for AFP and negative for Hep-Par-1. According to these histopathological findings, both tumors were diagnosed as HAC and metastatic HAC. The patient remained alive 16 mo after tumorectomy of the cerebral metastasis. The differential diagnosis of brain metastasis from metastatic tumors should use a panel of antibodies to avoid confusing with the brain metastasis of hepatocellular carcinoma (HCC). This paper describes this rare case of metastasis from gastric hepatoid adenocarcinoma to cerebral parenchyma, and provides a review of the literature concerning its histopathological and immunohistochemical characteristics.

INTRODUCTION

Gastric carcinoma is one of the most frequent tumors in the world. Its histological types include well- and poorly-differentiated adenocarcinoma, hepatoid adenocarcinoma (HAC), adenosquamous carcinoma, *etc*[1]. Metastasis of gastric carcinoma occurs mainly in organs and lymph nodes in venter imus, rarely in distant sites. Since Bourreille *et al*[2,3] reported the first case of gastric carcinoma with an increased level of serum alpha fetoprotein (AFP) in 1970, other cases have been reported[4,5]. In 1985, the term of hepatoid adenocarcinoma was used as malignant epithelial tumor derived from stomach by Ishikura *et al*[6]. The incidence rate of HAC is about 1.3%-1.5%. HAC produces a large amount of AFP, and is characterized by histopathological features, such as hepatoid differentiation, metastasis and poor prognosis. The number of brain metastases from gastric carcinoma, especially from gastric hepatoid adenocarcinoma is small. Only one case of cerebellar metastasis from gastric cancer with a higher level of AFP has been reported by Goda *et al*[7]. We present the first case of gastric hepatoid adenocarcinoma metastasis to cerebral parenchyma.

CASE REPORT

History and examination

A 50-year-old patient was admitted to our hospital with complaint of a one-month history of a paroxysm of headache in the left temple and pars parietalis accompanied with binocular caligation caligo, insensible feeling of limbs and transient anepia. The patient had no history of nausea and vomiting, spasm, palsy, aconuresis and copracrasia. Physical examination revealed decreased visual acuity and papilledema.

Radiological studies

Magnetic resonance (MR) imaging revealed a spherical occupying lesion of about 6.0 cm × 4.8 cm × 4.0 cm in

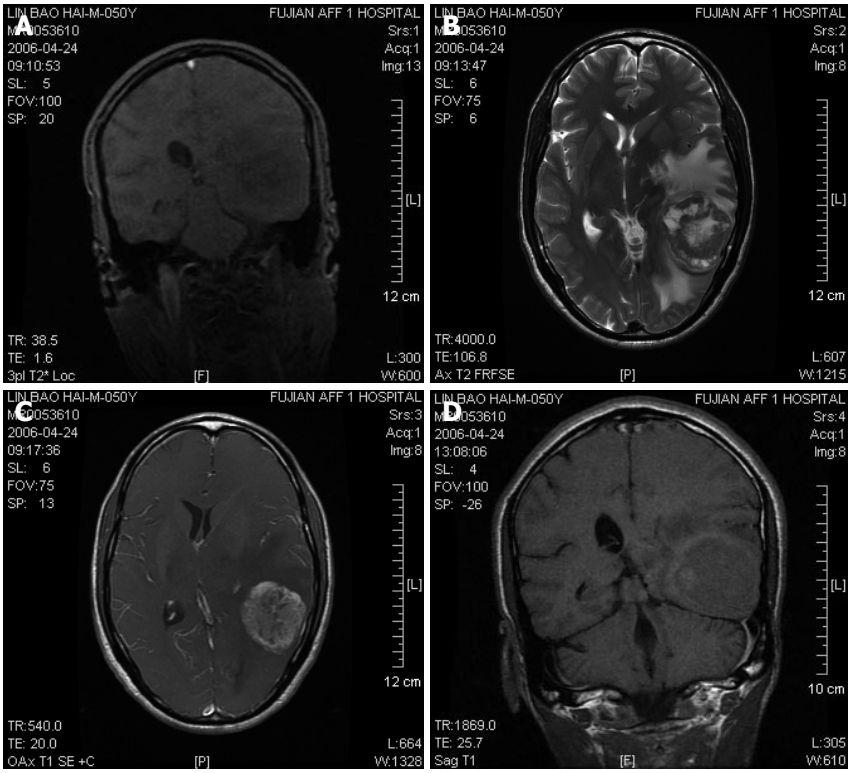

Figure 1 MR imaging revealing a spherical occupying lesion in the left posterior-temple lobe with a high intensity on T_2-weighted images and irregular contrast enhancement with a clear boundary and uneven signal in size of about 6.0 cm × 4.8 cm × 4.0 cm after administration of gadolinium-diethylene triamino pentaacetic acid (Gd-DTPA). **A**: Coronal gradient-recalled echo; **B**: Cross-sectional T2WI; **C**: Cross-sectional contrast enhancement T1WI; **D**: Coronal T1WI-delayed scan 4 h after contrast media injected *via* veins.

the left posterior-temple lobe with a high intensity on T_2-weighted images and irregular contrast enhancement with a clearly boundary and uneven signal after administration of gadolinium-diethylene triamino pentaacetic acid (Gd-DTPA). The lesion displayed an occupying effect, an out-inferior border close to the left transverse sinus, a perilesional irregular edema zone, compressed and deformed left posterior ventricle, and median line turned to dextroposition (Figure 1). The lesion was clinically diagnosed as a metastatic tumor.

Operation

On the 7th d after admission, the patient underwent tumorectomy. A tumor was found in subcortex of the left temporal lobe of about 0.3 cm and resected. A sample of about 4.0 cm × 5.0 cm × 5.0 cm was taken for pathological examination.

Pathological findings

Pathological analysis of the tumor sample revealed the growth pattern of solid cell nests. The polygonal tumor cells had abundant eosinophilic cytoplasm and round nuclei with obvious nucleoli. A pseudoglandular configuration containing proteic fluid was found in some parts of the tumor. Sinusoid-like blood spaces were located between nodular tumor cells (Figure 2). Metastasis of hepatocellular carcinoma was suspected.

Immunohistochemistry

Immunohistochemistry-stained hepatoid cells were positive for alpha-fetoprotein (AFP) and negative for Hep-Par-1 (Table 1, Figure 3), which did not match the immunohistochemical features of hepatocellular carcinoma metastasis.

Clinical history

Three years ago the patient was admitted to hospital due to recurrent pain in the epigastrium and accepted total gastrectomy because he was diagnosed having adenocarcinoma in cardia and corpora of the stomach with lymph node metastasis (Figure 4). No abnormal acoustic image in liver, pancreas, spleen and other organs was found on ultrasonograph. After surgery the patient received 6 mo of chemotherapy [calcium folinate (CF), 5-fluorouracil (5-FU) and cis-diamminedichloroplatinum (DDDP)]. The histopathological characteristics of brain tumor were identical to those of the sample sections. Immunohistochemistry-stained specimen of gastric carcinoma was positive for AFP and negative for Hep-Par-1 (Figure 5). Eight months after gastrectomy, an occupying lesion was detected in liver by ultrasound. CT scan also revealed a nodular or lump-like, high-density contrast mass in liver lobes during arterial phase, but the size of liver did not change, liver boundary was smooth and hepatic lobes were normal. The density of a larger mass of about 6.7 cm × 5.5 cm was uneven and the interior density was low at portal venous phase. The architecture of porta hepatis was clear (Figure 6). The diagnosis was liver metastasis from gastric carcinoma. Transcatheter arterial embolization for hepatic metastatic tumors with CF, 5-FU and DDDP was performed. The liver was rechecked by ultrasound every 6 mo after embolization. Before operation of the brain metastasis, ultrasound displayed no obvious abnormalities in liver and kidney and color Doppler flow imaging revealed no obvious bloodstream signals (Figure 7). Based on the results of histopathology and immunohistochemistry, the lesions were finally diagnosed as hepatoid adenocarcinoma (HAC) of cardia and corpora of the stomach and

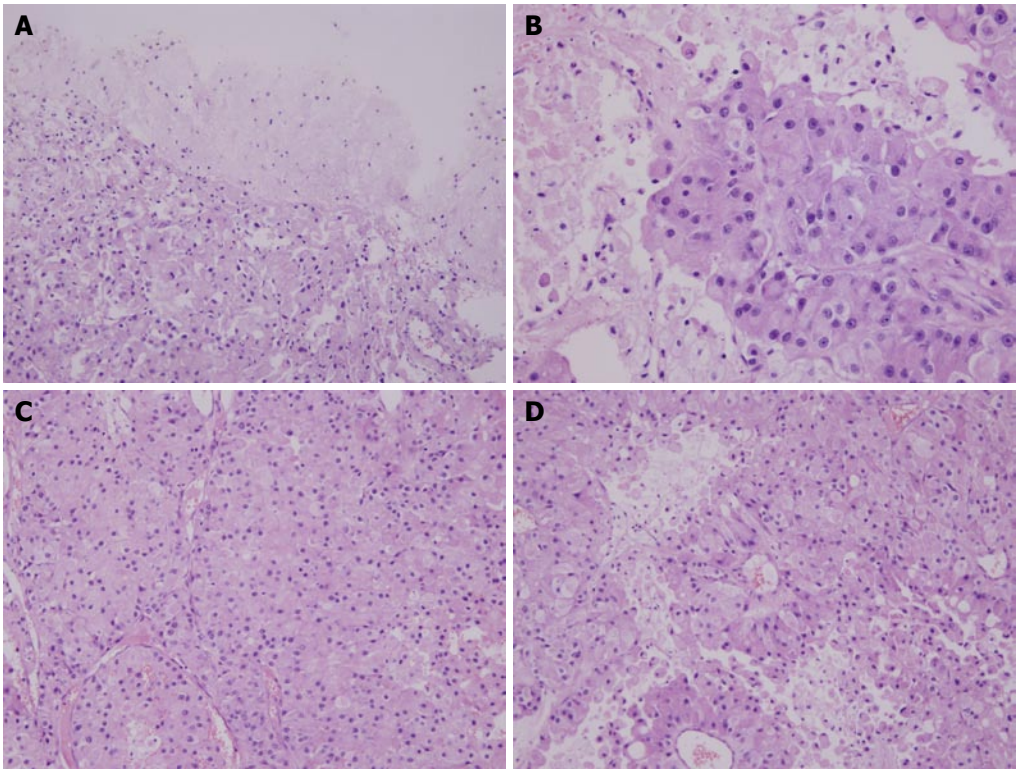

Figure 2 Brain metastatic tumor showing the growth pattern of solid cell nests (HE stain). **A**: Polygonal tumor cells with abundant eosinophilic cytoplasm, rich blood vessels and clear boundary of tumor and brain parenchyma (× 100); **B**: Polygonal tumor cells showing epitheliod and abundant eosinophilic cytoplasm, rich chromatin nuclei with obvious nucleoli (× 200); **C**: Round nuclei with obvious nucleoli, rich blood vessels resembling sinusoid-like blood spaces in hepatocellular carcinoma (× 200); **D**: Tumor cells exhibiting radial pattern surrounding thin- walled vessels (× 200).

metastatic hepatoid adenocarcinoma in the left posterior temple lobe, respectively. The patient was alive in the 16-mo follow-up period after operation of brain tumor.

DISCUSSION

Most patients with advanced gastric carcinoma die of metastasis and progressive cachexia. Metastasis of gastric carcinoma mainly occurs in peritoneal organs such as liver and lymph nodes; distant metastasis is rare. The incidence of intracranial metastasis from gastric carcinoma carcinoma ranges 1.3%-9.8%[8-10]. However, autopsy showed that the incidence of intracranial metastasis from gastric carcinoma is over 10.87%[11].

HAC is a special type of extrahepatic adenocarcinoma and its morphology is strikingly similar to that of hepatocellular carcinoma (HCC). Tumor cells consist of polygonal tumor cells with large central nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm, showing solid growth pattern and less trabecular and glandular as well as frequent lymphatic and vascular invasiveness, regional prominent mitoses, but no bile secretion and periodic acid-Schiff-positive diastase-resistant hyaline globules. HAC has been found in different organs, such as the stomach, lung, pancreas, esophagus, papilla of Vater, colon, bladder, kidney, ovary, uterus, cervix and peritoneal organs[4,5,12-21]. Most patients are males, ranging in age from 40 to 77 years. The antrum and pylorus are the frequent locations of gastric HAC. Most patients with HAC show an elevated AFP serum level. The clinical course of HAC patients is aggressive and poor in survival. The biological behavior of HAC is associated mainly with extensive hematogenous metastasis to the liver and early involvement of lymph nodes. Up to date, only one case of cerebellum metastasis from gastric carcinoma with an elevated AFP level has been reported[7]. However, no report on supratentorial cerebral parenchymal metastasis of gastric HAC is available.

Table 1 Immunohistochemical characteristics of cerebral metastasis and primary gastric carcinoma

	Cerebral metastasis	Primary gastric carcinoma
AE1/AE3	+	+
EMA	+	-
CK7	+	-
CK20	-	-
Villin	-	±
AFP	+	+
E-Cadherin	+	+
Hep-Par-1	-	-
CK8	+	+
CK19	+	+
34βE12	-	-
AACT	+	+
CD10	-	-
CD68	-	-
CK17	-	-
CK5/6	-	-
S-100	-	
GFAP	-	
SYN	-	
NSE	+	

Kodama *et al*[22] have reported two histological types of AFP-producing gastric carcinoma: medullary type and well-differentiated papillary or tubular type[22]. HAC of the medullary type is positive for AFP and well-differentiated adenocarcinomatous type is negative for AFP[6]. HAC of the medullary type has a striking morphologic similarity to HCC, and both of them produce high levels of AFP. Our patient had the morphology of the medullary type and was positive for AFP. Ishikura *et al*[6] suggested that this may be

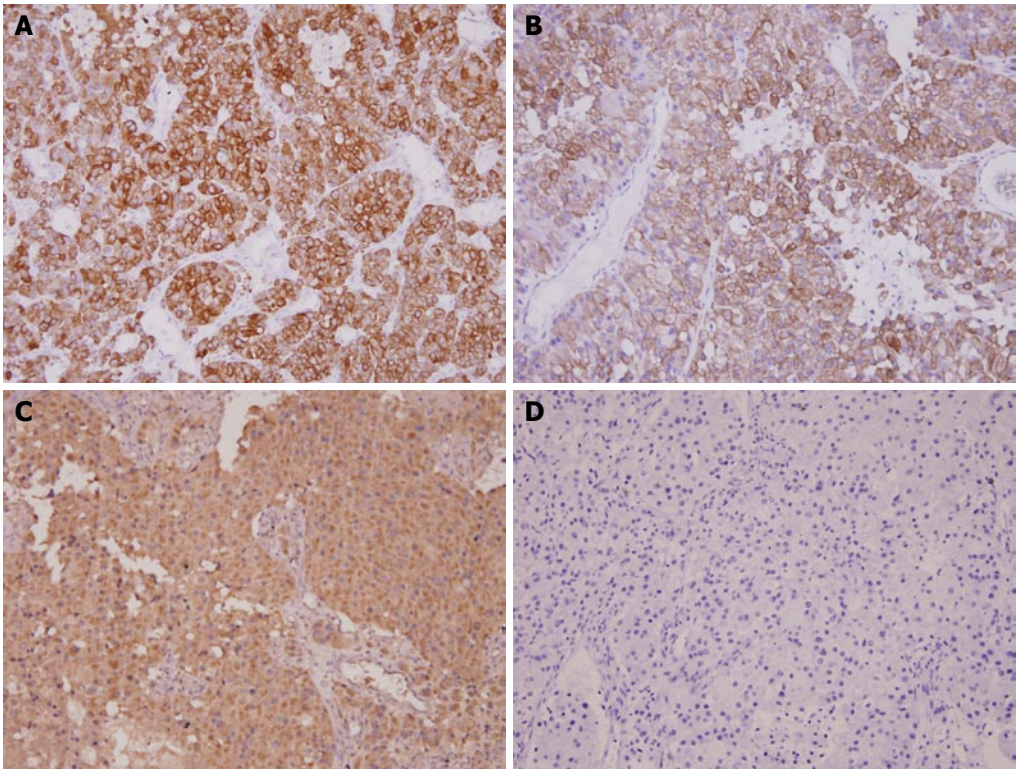

Figure 3 Immunoreactive characteristics of brain metastatic tumor (EnVision). **A**: Positively- stained cytokeratin AE1/AE3 (× 200); **B**: Positively-stained cytokeratin 8 (× 200); **C**: Positively-stained AFP (× 200); **D**: Negatively-stained Hep-Par-1 (× 200).

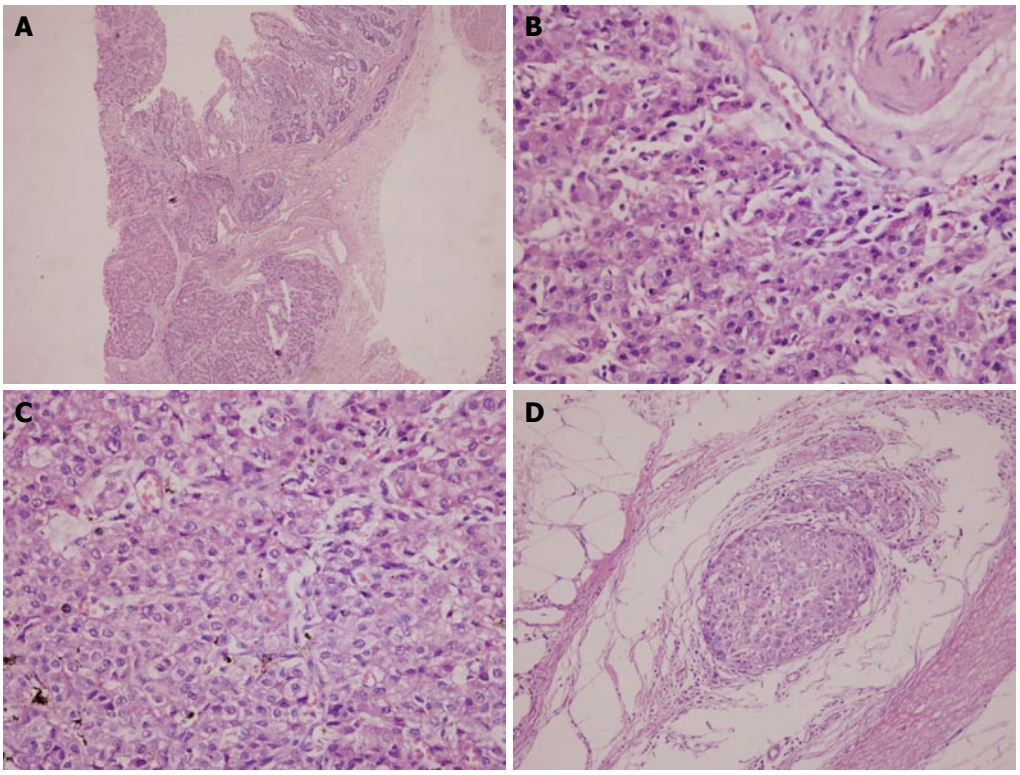

Figure 4 Histopathological features of primary gastric hepatoid adenocarcinoma. **A**: Tumor located at submucosa and intramuscle showing the growth pattern of solid cell nests (× 40); **B**: Part of tumor grown in cords separated by sinusoid-like blood spaces (× 200); **C**: Part of tumor grown in solid appearance showing polygonal epithelioid tumor cells with abundant eosinophilic cytoplasm, round nuclei with obvious nucleoli (× 200); **D**: Intralymph vascular tumor thrombosis in primary carcinoma (× 200).

due to the fact that the stomach and liver are both derived from primitive foregut of the embryo, and disturbances of differentiation may ultimately result in the development of foci of hepatocellular differentiation.

HAC and HCC share numerous clinicopathological features, such as elevated AFP serum level, hepatoid morphology and immunoreactivity with AFP, polyclonal carcinoembryonic antigen (CEA), and alpha-1 antitrypsin[23]. The histopathology of HAC and HCC is extremely similar, thus making the differential diagnosis difficulty, especially when the primary tumor is unknown. Immunohistochemistry of extrahepatic HAC and HCC is positive for AFP, CEA and cytokeratins 8 and 19, but not for cytokeratin 7[23,24]. The immunoreactivity of CK7 was positive in brain metastasis of our patient but negative in primary gastric carcinoma. The inconsistent phenomenon

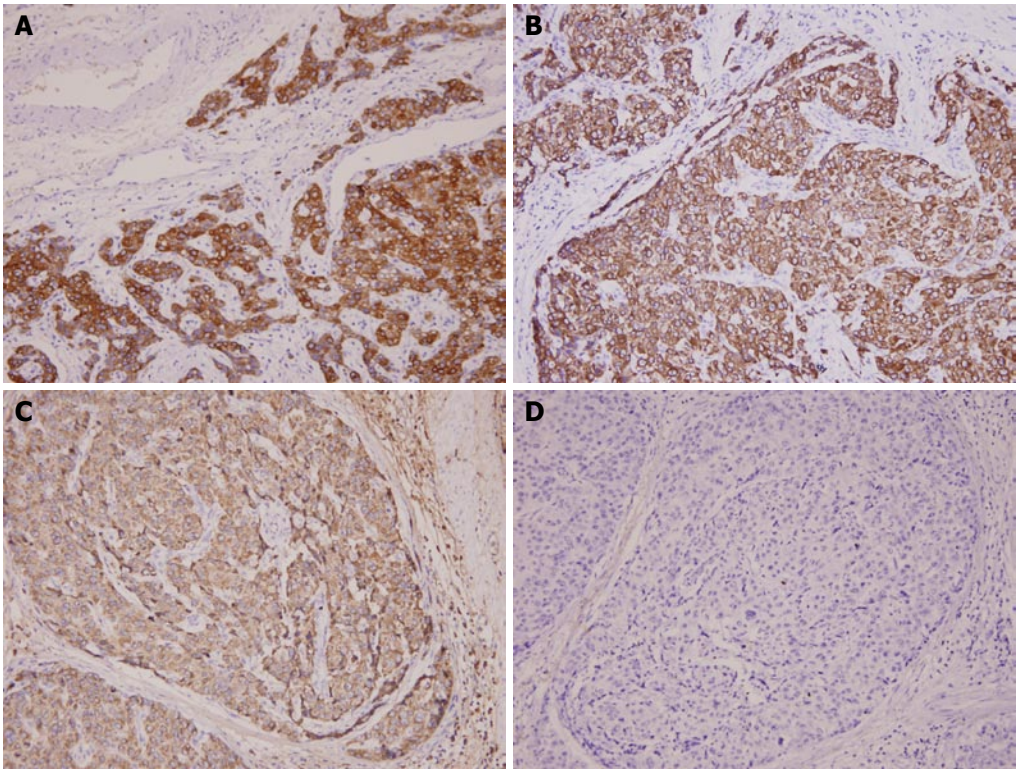

Figure 5 Immunohistochemical characteristics of primary gastric hepatoid adenocarcinoma. **A**: Positively-stained cytokeratin AE1/AE3 (× 200); **B**: Positively-stained cytokeratin 8 (× 200); **C**: Positively-stained AFP (× 200); **D**: Negatively-stained Hep-Par-1 (× 200).

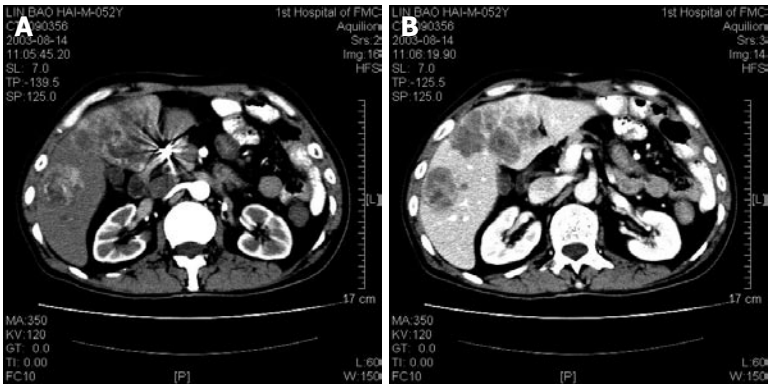

Figure 6 CT imaging features of liver metastatic tumor. **A**: CT scan revealing nodular or lump-like, high-density contrast mass in liver lobes during arterial phase; **B**: Uneven enhancement during portal venous phase.

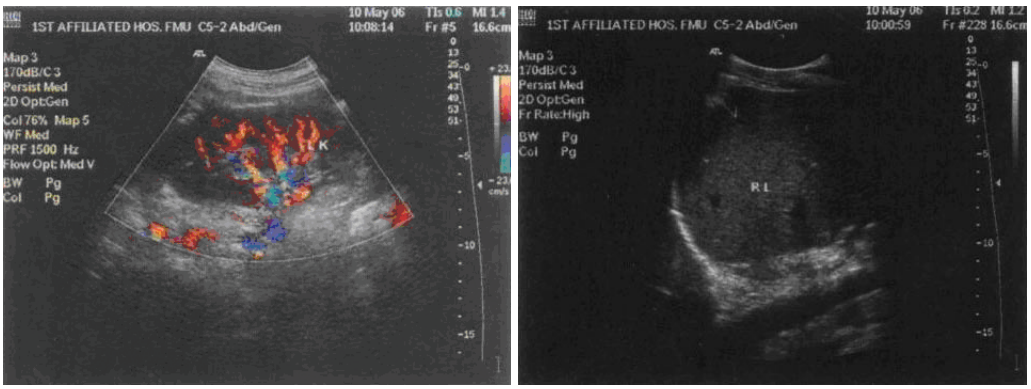

Figure 7 Features of ultrasound examination before operation of the brain metastasis.

of CK7 immunostaining is unable to explain. Therefore, it is uncertain that the brain metastasis is derived from liver metastasis from gastric HAC or from the primary gastric carcinoma.

Hep-Par-1 is a recently developed new monoclonal antibody that reacts with a hepatocyte-specific epitope. It was reported that the sensitivity and specificity of Hep-Par-1 in HCC are 82% and 90%, respectively[25], suggesting that this antibody is the most sensitive and specific immunohistochemical marker for hepatocyte differentiation[26-28]. In our patient, immunostaining showed that HCC was positive for Hep-Par-1 and extrahepatic

HAC was negative for Hep-Par-1.

Brain is one of the frequent hematogenous metastatic sites. It was reported that metastasis accounts for 13% of all brain tumors[9]. Usually, intracranial metastases and primary gliomas can be differentiated based on conventional MRI findings and clinical history. However, in some circumstances, especially when the lesion is solitary and its clinical features are nonspecific, conventional MRI alone is difficult to differentiate them. Although the management of metastases and primary gliomas is different, their morbidity and mortality are similar.

It was reported that the frequent symptoms of brain metastasis in recipients are headache, psychiatric symptom, hemicrania, vision disorder, cortical epilepsy[9]. The present patient had a similar clinical situation and intracranial metastases confirmed by MRI. Of the intracranial metastases, malignant melanomas account for 90%, chorioepithelioma malignum 60%, lung cancers 20%, breast cancers 16%, renal carcinomas 13% and thyroid carcinomas 9%. Brain metastasis of gastric cancer has been found only in about 0.5%-5% of cases reported, in which poorly-differentiated adenocarcinomas account for 27.3%-50%[9,29,30]. Most intracranial metastases from gastric cancer are lymphogenous and occur at meninges. Metastasis to the cerebral parenchyma *via* the arterial blood flow is rare. In general, patients with brain metastasis from gastric carcinoma have a higher stage of cancer and lymphovascular invasion or lymph node metastasis[9]. It is difficult to predict brain metastasis. If the patients have lymph node metastasis, lymphovascular invasion, or metastasis of other organs, they should be monitored carefully in order to detect brain metastases.

Treatment of brain metastasis depends mainly on whether it is solitary or multiple. Solitary metastasis is treated through resection, and multiple metastases are treated mainly with radiotherapy and chemotherapy. It was reported that multiple brain metastases account for 60%-70%, the prognosis of patients with brain metastasis is generally poor, and the mean postoperative survival time is about 6 mo[9]. The present patient remained alive 16 mo after the operation of brain metastasis. A longer survival time may be associated with the complete resection of brain metastasis and sensitivity to chemotherapy and radiotherapy.

In conclusion, metastasis of gastric hepatoid adenocarcinoma to the brain is rare. The differential diagnosis of metastatic tumors needs to use a panel of antibodies to avoid confusing with the brain metastasis of HCC. Long-term follow-up and close observation are required to find the symptoms of nervous system after gastrectomy, and early CT or MRI should be performed for the diagnosis of brain metastases. Solitary brain metastases are usually treated with surgery, radiotherapy and chemotherapy.

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Markus Reiser, Professor, PhD
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University of Colorado, Division of Gastroenterology, 4200 E. 9th Avenue, b-154, Denver, CO 80262, United Stetes

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Department of Internal Medicine, Konkuk University Hospital, Konkuk University School of Medicine, 4-12 Hwayang-dong, Gwangjin-gu, Seoul, 143-729, Korea

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Editorial Department of *World Journal of Gastroenterology*
77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

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Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com

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EDITORIAL

Potential role of NKT regulatory cell ligands for the treatment of immune mediated colitis

Madi El Haj, Ami Ben Ya'acov, Gadi Lalazar, Yaron Ilan

Madi El Haj, Ami Ben Ya'acov, Gadi Lalazar, Yaron Ilan, Liver Unit, Department of Medicine, Hebrew University-Hadassah Medical Center, Jerusalem, Israel
Correspondence to: Yaron Ilan, MD, Liver Unit, Department of Medicine, Hebrew University-Hadassah Medical Center, POB 12000, Jerusalem 91120, Israel. ilan@hadassah.org.il
Telephone: +972-2-6778231 Fax: +972-2-6431021
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Abstract

Natural killer T lymphocytes (NKT) have been implicated in the regulation of autoimmune processes in both mice and humans. In response to stimuli, this subset of cells rapidly produces large amounts of cytokines thereby provoking immune responses, including protection against autoimmune diseases. NKT cells are present in all lymphoid compartments, but are most abundant in the liver and bone marrow. They are activated by interaction of their T-cell receptor with glycolipids presented by CD1d, a nonpolymorphic, major histocompatibility complex class I-like molecule expressed by antigen presenting cells. Several possible ligands for NKT cells have recently been suggested. β-glucosylceramide, a naturally occurring glycolipid, is a metabolic intermediate in the anabolic and catabolic pathways of complex glycosphingolipids. Like other β-glycolipids, β-glucosylceramide has an immunomodulatory effect in several immune mediated disorders, including immune mediated colitis. Due to the broad impact that NKT cells have on the immune system, there is intense interest in understanding how NKT cells are stimulated and the extent to which NKT cell responses can be controlled. These novel ligands are currently being evaluated in animal models of colitis. Here, we discuss strategies to alter NKT lymphocyte function in various settings and the potential clinical applications of natural glycolipids.

Key words: Natural killer T lymphocyte; Immunomodulatory; Colitis; Inflammatory bowel disease; Ligand

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NKT REGULATORY LYMPHOCYTES

The term 'NKT cells' was first described in 1995[1] and defines a broad subset of mouse T-cells that share some characteristics with natural killer (NK cells), expression of the NK1.1 marker in particular. This is a heterogeneous subset of lymphocytes some of which do not express the NK1.1 marker[2]. NKT cells develop from thymocyte progenitor cells similarly to conventional T-cells. However, unlike conventional T-cells, NKT cells express a T-cell receptor (TCR) that recognizes glycolipids rather than protein antigens[3]. The largest subset of NKT cells expresses a highly restricted TCR comprised of an invariant TCR α chain with a single rearrangement (in mice Vα14-Jα18, and in humans Vα24-Jα18)[4] coupled with TCR β chains with limited heterogeneity due to marked skewing of Vβ gene usage (mostly Vβ8.2 in mice and Vβ11 in humans)[5]. This population, also referred to as invariant NKT cells (iNKT), is highly conserved in most mammals studied to date. iNKT cells are restricted by the major histocompatibility complex (MHC) class I-like molecule CD1d, which is expressed by conventional antigen presenting cells (APCs) including macrophages, dendritic cells, and marginal zone B cells[2].

CD1d-mediated glycolipid presentation to NKT cells is an important aspect of immune regulation. However, as an illustration of NKT complexity, there is a type of NKT-cell that expresses the NK1.1 marker, but is CD1d independent. There are two broad classes of cells that satisfy the criteria of being CD1d dependent NKT cells. For the purposes of this review, we classify these as type I NKT cells, being the Vα14-Jα18 (mouse) or Vα24-Jα18 (human) population, and type II NKT cells, which includes all other CD1d-dependent T cells[6].

The inherent, low-level auto-reactivity of certain specialized immune cell types that have both innate and adaptive characteristics, such as CD1d restricted NKT cells, γδ T cells, and B1 cells, suggests that these cell types may also have the potential to stimulate autoimmunity[2]. Activation of iNKT cells occurs early in a number of microbial infection models in mice, and such activation can lead to reinforcement of the innate immunity and promote subsequent adaptive immunity. Thus, immune responses to certain bacterial, viral, and parasitic infections and tumors can be enhanced whereas autoimmune disease and allograft rejection can be suppressed[5].

THE ROLE OF NKT CELLS IN IMMUNE RESPONSES

NKT cell Th1 and Th2 responses can offset one another;

therefore, polarizing cytokine release toward either one may serve as an important therapeutic tool[7]. These lymphocytes constitutively express cytokine mRNA, and within hours of activation produce large amounts of cytokines such as IFN-γ, TNF, IL-4, and IL-10[5]. NKT cell-mediated regulation of immune responses has been demonstrated to influence a large number of disease states[5]. These cells have received considerable attention in recent years as innate lymphocytes that can modulate T-cell and APC functions in autoimmunity. A potential link between NKT cells and autoimmunity was suggested by the finding that various mouse strains, including non-obese diabetic (NOD) mice that are genetically susceptible to autoimmunity[8,9], have a reduced number and defective function of iNKT cells as compared with non-autoimmune mouse strains[10]. Diminished numbers of NKT cells have been correlated with an increased incidence of autoimmune diseases including systemic lupus erythematosus, scleroderma, type I diabetes, multiple sclerosis, and rheumatoid arthritis[11-16].

The adoptive transfer of NKT cells has ameliorated disease in several immune-mediated animal models, including experimental autoimmune encephalomyelitis[17], immune mediated colitis[18], and graft versus host disease (GVHD)[19]. In addition, NKT lymphocytes play an important role in diverse neoplastic and infectious processes, and as such may serve as a target for potential new immune-therapeutic strategies[20,21]. NKT cells are now known to be a major source of IFN-γ, which is required for early activation of macrophage bactericidal activity[22]. Several studies have demonstrated a role for NKT lymphocytes in anti-tumor immunity[23]. Mouse and human NKT cells were shown to exert cytotoxic activity towards several tumor cell lines[24]. NKT lymphocytes were found to promote tumor rejection in experimental models of tumor immunotherapy by administration of IL-12 or α-GalCer[25]. In a murine hepatocellular carcinoma (HCC) model, NKT cells were shown to have a role in oral immune regulation with HCC lysate and HBV envelope proteins, and in adoptive transfer of dendritic cells pulsed *ex vivo* with the same antigens[20].

LIGANDS FOR NKT REGULATORY CELLS

Through their semi-invariant TCR, NKT cells recognize glycolipids presented in the context of the CD1d molecule[26]. CD1 proteins are a family of molecules that have structural homology to MHC class I molecules, but are unusual in their ability to present glycolipid antigens to T-cells[27]. Because NKT cells can produce cytokines that result in conflicting responses, the possibility exists that the ligand structure can polarize NKT cell responses toward either a Th1 or a Th2 response[28].

Glycosphingolipids, or glycolipids, are a family of both naturally occurring and synthetic molecules composed of a hydrophobic ceramide backbone, N-acylsphingosine, and a hydrophilic head group made of carbohydrates, mono- or oligosaccharides[29]. Enzymatic defects and subsequent accumulation of certain glycolipids can lead to "storage" diseases such as metachromatic leukodystrophy, Gaucher's or Fabry's disease[30]. Patients with Gaucher's disease have altered humoral and cellular immune profiles[31] and increased peripheral blood NKT lymphocytes[32]. In the context of stimulatory glycolipids, an understanding of how glycolipid structure affects cytokine release profiles is essential.

α-galactosylceramide (α-GalCer) was originally discovered during a screen for reagents derived from the marine sponge *Agelas mauritianus* that prevented tumor metastasis in mice[33]. KRN7000, the synthetic α-GalCer analogue, is a high-affinity ligand for the CD1d molecule[34]. *In vivo* administration of α-GalCer to mice or humans results in rapid and robust cytokine secretion by iNKT cells, followed by the activation of a variety of cell types of the innate and adaptive immune systems[35].

OCH is a truncated analogue of α-GalCer in which the sphingosine chain has been shortened from 18 to 9 carbons. Following its administration to mice, the early production of IL-4 by NKT cells remained intact while the bulk of IFN-γ, mostly derived from NK cells, was lost, leading to a Th-2 biased response[36]. The ratio of IL-4 to IFN-γ released by NKT cells is influenced by the length of the lipid chain; shorter chain lengths increase this ratio[3]. Administration of α-C-GalCer leads to a strong Th-1 biased response with sustained IFN-γ levels for several days compared to the 24-h response induced by α-GalCer[37]. Treatment with α-C-GalCer was more potent than α-GalCer in mouse models of malaria and malignant tumors, while treatment with OCH was more efficacious than α-GalCer in the Th-1 mediated autoimmune disease models of encephalomyelitis and colitis[38].

Activation of NKT cells *via* α-GalCer has been shown to affect numerous models of malignancy, infection, and autoimmune disease[3]. In models with strong NKT cell involvement, such as in type I diabetes-prone NOD mice, activation of NKT cells with α-GalCer delayed disease induction and prevented its recurrence[39,40]. On the other hand, treatment with α-GalCer can cause disease exacerbation, an effect noted mainly in models where these molecules play a "pathogenic" role such as in the F1 mouse model of lupus nephritis (NZB × NZW)[41], or the apolipoprotein E knockout mouse model of atherosclerosis[42,43]. Despite their promising effects in diverse disease situations, the clinical use of α-glycolipids has been limited by their side effects, mainly hepatotoxicity[44,45].

NATURAL LIGANDS FOR NKT CELLS

The discovery of the marine sponge-derived glycolipids as ligands for NKT cells led to studies looking for possible natural ligands. These natural antigens can be separated into two groups: (1) antigens that are produced by the host (endogenous antigens), and (2) antigens from foreign pathogens (exogenous antigens). The strongest evidence for the presence of an endogenous antigen is that positive selection of NKT cells in the thymus requires presentation of an antigen recognized by the TCR[3]. The best evidence for the presence of exogenous antigens is that antigen presentation proteins related to CD1d have been characterized as presenters of microbial glycolipids, and it was speculated that NKT cells might survey for the

presence of infectious agents[46-48].

Given the auto-reactivity of the NKT TCR to CD1d and the limited diversity of TCRs that NKT cells express, it is generally accepted that a single, or set of closely related, autologous glycolipid ligands are responsible for the activation of these cells. These endogenous ligands have yet to be identified. Recently, the lysosomal glycolipid, isoglobotrihexosylceramide (iGb3) has been proposed as a natural ligand for NKT cells[49]. This beta structured-glycolipid, in its natural or synthetic forms, has the ability to activate most human or mouse NKT cells *in vitro*. Impaired generation of lysosomal iGb3 in mice lacking β-hexosaminidase *b* resulted in severe NKT cell deficiency, suggesting a role for iGb3 in murine NKT cell development[49]. Recently, some NKT cell activating antigens of microbial origin have been found[50]. NKT cells have been found to play a role in controlling infection by organisms such as *Mycobacterium tuberculosis* where NKT cells predominate in the anti-mycobacterial granulomatous reaction[51,52], *Plasmodium bergehi*, *Listeria monocytogenes*[53], *Ehrlichia muris*, and *Sphingomonas capsulata*[54].

At least two mechanisms have been proposed for NKT cell activation. The first is "enhanced auto reactivity", where APC recognition of microbial antigen results in IL-12 mediated APC-NKT cell activation. The second is a CD1d presented microbial glycolipid that triggers iNKT cells through TCR recognition[2,3]. There has been some success in identifying specific microbial glycolipid ligands of CD1d that can activate NKT cells, most notably, α-glycuronosylceramides (α-galacturonosyl and α-glucuronosylceramide) derived from the lipopolysaccharide-negative *Sphingomonas* bacteria cell wall[55]. These α-glycuronosylceramides are of specific significance because they share structural homology with α-GalCer. Other examples include the CD1-restricted presentation of *Plasmodium berghei* sporozoite-derived GPI anchor that stimulates NKT-cell-mediated B-cell activation and antibody production[56], and the phosphatidylinositol tetramannoside (PIM4) produced by *Mycobacterium bovis*[57]. These activities suggest a role for NKT cells in the innate response against pathogens that do not activate classical pattern-recognition receptors, such as Toll-like receptor 4.

β-GLYCOLIPIDS AS NKT LIGANDS

Recent studies have shown that different glycolipids preferentially target different organelles. Because different isoforms of CD1 localize to different subcellular compartments, they allow APCs to present a variety of glycolipid antigens that enter the cell by different pathways and are targeted to different locations[58]. β-glycolipids are naturally occurring intermediates in the anabolic and catabolic pathways of complex glycosphingolipids and are found in cell membranes[59]. Past studies have suggested that β-glycolipids do not possess stimulatory properties on NKT cells[59]. However, recent data have suggested that these compounds may have an important NKT cell mediated immune modulatory effect. β-glucosylceramide (GC) is a beta glycolipid that is degraded into ceramide by glucocerebrosidase. CD1d-bound GC does not stimulate NKT cells directly[60]. β-glycolipids may inhibit NKT activation and even block the stimulatory effect of α-GalCer on these cells. Glucosylceramide-synthase deficiency leads to defective ligand presentation by CD1d, with secondary inhibition of NKT cell activation[60]. *In vitro*, administration of GC led to a 42% decrease in NKT cell proliferation in the presence of DCs, but not in their absence[61]. Additional naturally occurring β-glycolipids such as β-lactosylceramide (LC) and β-galactosylceramide (GLC) are being tested for their immunomodulatory effects (unpublished data).

Administration of β-glycolipids in several Th1 mediated disease models such as auto-immune hepatitis, metabolic syndrome, and acute GVHD, alleviated the disease while inducing a Th2 cytokine profile[61-63]. In a murine model of concanavalin A-induced hepatitis, administration of GC led to significant amelioration of liver damage[61]. This beneficial effect was associated with a 20% decrease in intrahepatic NKT lymphocytes, a significant lowering of serum IFN-γ levels, and decreased STAT-1 and STAT-6 expression. The administration of GC to leptin-deficient ob/ob mice, an NKT dependent model, significantly improved the metabolic alterations[62]. Liver fat content was reduced significantly in both MRI and histological examinations. In addition, treated mice achieved near-normalization of glucose tolerance and decreased serum triglyceride levels. These effects have been associated with a marked increase of the peripheral/intrahepatic NKT cell ratio. In a semi-allogeneic model of acute GVHD, GC-treated mice manifested a significant decrease in skin, bowel, and liver GVHD manifestations[64]. The beneficial effect of GC was associated with decreased IFN-γ and increased serum IL-4 levels, as well as a significant increase in the intrahepatic to peripheral NKT lymphocyte ratio and in intrahepatic $CD8^+$ lymphocyte trapping[64]. In contrast, in Th2 mediated models of disease, administration of β-glycolipids also led to NKT mediated disease alleviation associated with an opposite Th1 immune shift. In a murine model of hepatocellular carcinoma, GC led to improved survival rates and a decreased tumor volume[63]. These effects have been associated with an 11-fold increase in intrahepatic NKT lymphocyte number. Taken together, these results suggest that certain β-glycolipids may serve as a "fine tuners" for NKT lymphocyte-mediated immune responses and may have a beneficial effect in seemingly opposing disease models.

NKT CELLS IN INFLAMMATORY BOWEL DISEASE

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract that are associated with an imbalance between Th1 pro-inflammatory and Th2 anti-inflammatory subtypes of immune responses. The abundance of CD1d-positive cells in the human intestine suggests a role for these cells in chronic inflammatory disorders of the bowel. NKT cells have been proposed to make both protective and pathogenic contributions to IBD[65]. Ulcerative colitis (UC) is a subtype of IBD that is limited to the superficial

layers of the colon and is dominated by the production of Th2 cytokines. Studies have shown that classical (type I) CD1d-restricted NKT cells contribute to a murine model for UC[66,67]. NKT cells exerted protective effects against DSS colitis, a model for intestinal inflammation that primarily targets mucosal macrophages. In this model, administration of α-GalCer and adoptive transfer of NKT cells resulted in reduction of inflammation.

The role of NKT cells in chronic bowel inflammation is complex. They can play either a protective or a pathogenic role in intestinal inflammation, depending on the type of inflammatory process and the antigen presented in the gut. NKT cells support a pro-inflammatory immune response in TNBS-colitis, a Th1 model. Thus, depletion of NKT cells results in alleviation of the disease[68], effects which were mediated by altered intrahepatic $CD8^+$ trapping and that increased INF-γ producing lymphocytes[69]. Feeding colitis-extracted proteins (CEP) to mice with TNBS-induced colitis induces oral tolerance and alleviates TNBS-induced colitis[70]; NKT depletion prevents oral tolerance induction18. Adoptive transfer of *ex vivo* CEP-pulsed NKT cells also alleviated colitis[69]. NKT cells exerted protective effects against DSS colitis, a model for intestinal inflammation (Th2 model) that primarily targets mucosal macrophages. In this model, administration of α-GalCer and adoptive transfer of NKT cells reduced inflammation. In contrast, oxazlone-colitis could not be induced in animals lacking NKT[71]. Several studies proposed a role for NKT cell activation in IBD patients. Expression of CD1d is higher in the epithelia of the affected terminal ilea of CD patients and in the affected cecum of UC patients, which may lead to recruitment of proinflammatory CD1d-reactive cells from the periphery, resulting in mucosal destruction[72]. However, a more recent report suggested that, in contrast to normal colon surface epithelium, epithelial cells derived from UC or CD patients do not express CD1d[73]. The diminished expression of CD1d was suggested as a possible mechanism for impaired regulatory NKT cell function in IBD. Taken together, these data suggest a complex role for NKT cells in chronic inflammatory disorders of the bowel, which may involve various factors in the immune microenvironment.

EFFECT OF NKT LIGANDS IN ANIMAL MODELS OF IMMUNE MEDIATED COLITIS

Experimental colitis induced by intracolonic installation of TNBS, is associated with a Th-1 immune response as evidenced by increased IFN-γ secretion, decreased IL-10 and IL-4 secretion, and reduction in the intrahepatic $CD8^+$ trapping. These effects were hypothesized to be mediated by regulatory NKT cells[69]. Several glycolipid derivatives have been shown to alleviate hapten mediated colitis. OCH, and α-Gal-Cer analogue with truncated sphingosine chain, attenuates colonic inflammation as defined by body weights and histological injury[38]. The protective effects could not be observed in Vα14 NKT cell-deficient mice, further evidence of an NKT role in the pathogenesis of colitis. The immunomodulatory effect of several β-glycolipids, including GC (glucosylceramide), LC (lactosylceramide), GLC (galactosylceramide), and IGL (GC + LC), was shown to be associated with increased survival and significant alleviation of colitis with improvement in the macroscopic and microscopic scores[63]. Administration of GC alleviated immune mediated experimental colitis, improving both the macroscopic and microscopic scores. The beneficial effects of GC were associated with an increased peripheral/intrahepatic CD4/CD8 lymphocyte ratio and a Th2 immune shift.

In summary, NKT cells may make both protective and pathogenic contributions to IBD[65]. Studies show that these cells are involved in the maintenance of mucosal homeostasis. On the other hand, this subset of cells plays a pathogenic role in human ulcerative colitis. Similar contrasting data have been generated in murine models of IBD[65]. Whether the apparent differences in NKT response patterns depends on variations in NKT ligands and/or on the presence of specific subsets of mucosal NKT cells remains to be elucidated. Further studies that determine the subset of NKT cells and the specific ligands involved in these disorders may facilitate the development of novel therapies for IBD.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Rectal cancer treatment: Improving the picture

Juan A Diaz-Gonzalez, Leire Arbea, Javier Aristu

Juan A Diaz-Gonzalez, Leire Arbea, Javier Aristu, Department of Oncology, Clinica Universitaria, Universtity of Navarra, Pamplona 31080, Spain
Correspondence to: Juan A Diaz-Gonzalez, Department of Oncology, Clinica Universitaria, Universtity of Navarra, Avda. Pio XII, 36, Pamplona 31080, Spain. jadiaz@unav.es
Telephone: +34-948-255400 Fax: +34-948-255500
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Abstract

Multidisciplinary approach for rectal cancer treatment is currently well defined. Nevertheless, new and promising advances are enriching the portrait. Since the US NIH Consensus in the early 90's some new characters have been added. A bird's-eye view along the last decade shows the main milestones in the development of rectal cancer treatment protocols. New drugs, in combination with radiotherapy are being tested to increase response and tumor control outcomes. However, therapeutic intensity is often associated with toxicity. Thus, innovative strategies are needed to create a better-balanced therapeutic ratio. Molecular targeted therapies and improved technology for delivering radiotherapy respond to the need for accuracy and precision in rectal cancer treatment.

Key words: Rectal cancer; Chemoradiotherapy; Intensity-modulated radiation therapy; Molecular targeted therapy

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WHERE WE ARE: INTRODUCING THE CHARACTERS

Since the early 90's, radical surgery and fluoropirimidine-based chemoradiotherapy (CHRT) are the gold standards of treatment for locally advanced rectal cancer. Studies conducted by the Gastrointestinal Tumor Study Group[1,2] and the North Central Cancer Treatment Group[3] concluded that the combination of postoperative chemotherapy with radiotherapy improved local tumor control and survival in stage Ⅱ and Ⅲ rectal cancer relative to surgery alone.

Although currently the big picture mostly remains, some of the characters of the puzzle have changed. The main milestones in this development began with the improvement of the surgical technique, total mesorectal excision (TME). TME became the choice surgical procedure, with a relevant increase in local control. Actually, at some point it was thought that TME could make radiotherapy (RT) unnecessary. Nevertheless, a randomized study soon followed showing the maintained benefit of RT despite an excellent surgery, at least in terms of local control[4], outcomes that even are improving with longer follow-up.

The second landmark was to move the CHRT segment before the surgery. Initially, preoperative radiotherapy was found to improve overall survival as compared with surgery alone[5,6]. In the last decade, the dominant tendency in the therapeutic development of rectal cancer, both in Europe and North America, has been the use of preoperative radiotherapy with conventional protracted fractionation (45-50 Gy in daily fractions of 1.8-2 Gy during 5-6 wk) with concurrent chemotherapy followed by surgery at 4-8 wk. Extensive experience with preoperative CHRT showed feasibility and promising results in terms of down staging, sphincter preservation and disease control and survival parameters as interesting elements of analysis, with an acceptable toxicity profile. The most frequently used chemotherapy agent in this clinical context is 5-fluorouracil (5-FU, i.v.)[7-13]. More recently, the only phase Ⅲ trial concluded comparing pre- *vs* post-operative CHRT, demonstrated better tolerance, sphincter-saving surgical procedures and local control with preoperative CHRT[14].

Preoperative radiotherapy alone (no chemotherapy) and delayed surgery reported down staging rates of 18%[15,16]. However, the prolonged administration of CH-RT achieves down staging figures of around 65%[7-11,17]. Additionally, induction of tumor down staging improves the probability of a complete resection and sphincter-preserving surgery[11,13,18-20].

Complete pathologic response (pCR) rates range from 8% to 27% using i.v. 5-FU with preoperative irradiation[7,10,11,14,21]. In studies of postoperative 5-FU-based CHRT, severe acute toxicity ranges from 24%-40%[1,14,22,23]. However, in Phase Ⅱ studies of preoperative CH-RT, Grade 3-4 acute toxicity occurs in 15%-28% of patients[7,11,13,14,20].

Regarding tumor control and survival, published series

Table 1 Novel chemoradiation combinations

	Chemotherapy		RT (Gy)	GI grade 3-4 toxicity (%)	DS (%)	pCR (%)
	Capecitabine (mg/m^2 bid)					
Kim *et al*	825 d 1-14 and 22 - 35		50.4	-	84	31
De Paoli *et al*	825 bid continuous		50.4	-	57	24
	5-FU (mg/m^2 CI)	CPT-11 (mg/m^2 weekly)				
Mehta *et al*	200	50	50.4	28	71	37
Klautke *et al*	250	40	50.4	32	76	24
Mohiuddin *et al*	Arm 1: 225	Arm 1: -	HART: 55.2-60	27	78	26
	Arm 2: 225	Arm 2: 50	50-54	37	78	26
Navarro *et al*	225	50	45	14	49	14
	5-FU (mg/m^2 CI)	Oxaliplatin (mg/m^2)				
Ryan *et al*	200	MTD: 60 weekly	50.4	38	-	25
Aschele *et al*	200-225	MTD: 60 weekly	50.4	16	84	28
Turrito *et al*	300	80 wk 1, 3, 5	45	-	65	15
	Capecitabine (mg/m^2 bid)	Oxaliplatin (mg/m^2)				
Rodel *et al*	825 d 1-14 and 22 - 35	50 d 1, 8, 22	50.4	6	55	19
Machiels *et al*	825 bid continuous	50 weekly	45	30	-	14

RT: Radiotherapy; DS: Downstaging; bid:Twice daily; CI: Continuous infusion; HART: Hyperfractionated accelerated radiotherapy; MTD: Maximun-tolerated-dose; GI: Gastrointestinal.

vary in follow-up. Preoperative CHRT in rectal cancer assumes ranges for 5-year local recurrence from 2% to 15%, disease-free survival from 70% to 86%, and overall survival from 60% to 85%[7,9,10,14,18,21,24-26].

In summary, incorporation of TME surgical procedure and 5-FU-based preoperative CHRT have been translated to an improvement in local control, with the additional advantage of more tolerable treatments in terms of acute toxicity and saving-sphincter surgical procedures.

MOVING FORWARD: IMPROVING THE PORTRAIT

The picture is drawn. What is next, more characters or better colors?

Therapeutic intensity is often linked to better response and outcomes. But in oncology more is not always better. Increases in doses or number of therapeutic agents combined together lead to higher rates of toxicity. This situation is especially true in rectal cancer. Moreover, the risk of over-treatment in some patients with rectal cancer is present. One treatment approach for all rectal adjuvant patients may not be warranted. We already know that not every stage Ⅱ-Ⅲ rectal cancer is the same[27]. Prognostic factors have been studyed, both at clinical and at molecular and genetic level. In the near future these signatures should be taken into account. An adequate therapeutic index should be found, with a well-balanced ratio of benefit/toxicity.

Where can we find additional benefit in rectal cancer treatment? On the one hand, despite the improvement in local control with multimodality approaches, the rate of distant metastasis is still high, around 19%-36%[10,14]. On the other hand, growing data demonstrates a relationship between response to preoperative CHRT and survival. A higher grade of tumor regression in the surgical specimen has been associated with increased disease-free survival and overall survival after preoperative CHRT in rectal cancer[10,24,17,28-31]. Thus, achieving higher rates of complete pathologic response, but also major tumor regression, is one of the current goals in the protocols of preoperative CHRT in rectal cancer. Both effects, reduction of distant metastasis and higher tumor regression grade, require the use of more active and effective chemotherapeutics agents, with adequate toxicity profiles when administered with radiotherapy.

Exploring novel CHRT combinations

Oral fluoropyrimidines: Oral fluoropyrimidines have been developed as a therapeutic alternative to i.v., continuous infusion of 5-FU, and have been shown to deliver similar efficacy and tolerability with the additional advantage of offering the convenience of oral chemotherapy (Table 1).

Few studies have investigated the safety and efficacy of tegafur with or without uracile (5-FU pro-drugs) and radiotherapy[32-35]. Down staging rates (54%-68%), pCR (8%-15%), and grade 3-4 toxicity (12%-43%) match quite well with those with i.v. 5-FU. Although follow-up is not as long as in the 5-FU series, outcomes in terms of local control, distant metastasis rate, disease-free survival and overall survival seem to be similar.

Capecitabine is a fluoropyrimidine carbamate active

in several solid tumors. A recent phase Ⅲ trial (X-ACT trial) has demonstrated the equivalence of capecitabine to bolus 5-FU/leucovorin in the adjuvant treatment of colon cancer[36]. Thymidine phosphorylase (TP) is a key enzyme for the metabolism of capecitabine to 5-FU. Some data suggest that tumor tissue shows higher concentrations of TP than normal tissue[37]. This phenomenon would lead to a preferential activation of capecitabine in the tumor tissue, providing a favorable ratio for toxicity and radiosensitization. Preclinical studies have shown that RT might up-regulate the TP expression in tumor cells, resulting in a selective and synergistic effect between RT and capecitabine[38]. Phase I studies have been conducted to determine the maximun-tolerated-dose (MTD) of capecitabine in combination with radiotherapy. The recommended dose for this combination was 825 mg/m^2 bid without break during radiotherapy period (5-6 wk)[39,40]. Two published phase Ⅱ studies have shown that preoperative CHRT with capecitabine appears to be effective in locally advanced, resectable rectal cancer. Encouraging rates of down staging (up to 84%) and pCR (24%-31%) with a favorable safety profile of the combination might warrant the use of capecitabine and RT with other effective new drugs[40-42].

Irinotecan (CPT-11): Irinotecan is an active chemotherapeutic agent in colorectal cancer. The combination of Irinotecan and 5-FU has been approved as first line chemotherapy for patients with metastatic colorectal cancer[41,43,44]. Phase I studies have demonstrated that CPT-11 can be safely administered concomitantly with radiotherapy (MTD: 10 mg/m^2 daily or 50 mg/m^2 weekly)[45]. Several phase Ⅱ studies have determined the efficacy and feasibility of the irinotecan and 5-FU combined-therapy plus radiotherapy in the neo-adjuvant management of rectal cancer. The rates of tumor down staging (49%-78%) and pCR are high (14%-37%) with an acceptable rate of acute severe toxicity (14%-37%)[46-49].

The combination of CPT-11 and Capecitabine with radiotherapy has been studied in recent phase Ⅰ-Ⅱ trials[50,51]. The MTD dose of Capecitabine was 500 mg/m^2 while combining with CPT-11 50 mg/m^2 weekly and 750 mg/m^2 while combining whit CPT-11 40 mg/m^2 weekly. The rate of tumor down staging and pCR were similar with the two schedules (72%-75% and 14%-21%, respectively) and similar with the combination of 5-FU, CPT-11 and radiotherapy.

Oxaliplatin: Oxaliplatin is a novel anti-neoplastic platinum. When combined with 5-FU, oxaliplatin improves overall survival for patients with metastatic colorectal cancer and the rate of progression-free survival for patients with completely resected stage Ⅱ and Ⅲ colon cancer[52,53]. These data encourage combining oxaliplatin and 5-FU in the preoperative setting of rectal cancer management for an improved response. Moreover, oxaliplatin has radiation sensitization properties[54].

Several phase Ⅱ studies have evaluated weekly administration schedules of oxaliplatin and 5-FU and radiotherapy. They have demonstrated that this regimen is feasible with moderate toxicity. The addition of oxaliplatin to standard 5-FU-RT seems to be associated with a promising down staging (65%-84%) and pCR rates (15%-28%)[55-57].

Oxaliplatin has been combined with Capecitabine in metastatic colorectal disease[58-60]. The combination has been adapted to preoperative CHRT and phase Ⅰ-Ⅱ trials have been published. The studies show that this regimen is active and feasible, with attractive down staging (55%-72%) and pCR rates (14%-28%)[61-63].

RAISING THE BAR: THERAPEUTIC MODULATION

One of the paradigms for loco regional treatment of cancer is anatomic precision. Technical advances in radiation oncology including functional and molecular imaging and intensity-modulated radiation therapy (IMRT) delivery techniques are allowing greater treatment precision and dose escalation. Moreover, cancer is a biologic entity. Treating cancer requires understanding cancer biology which is changing the approach in cancer therapeutics. A number of genetic signatures and molecular pathways involved in cancer have been discovered. Parallel molecular therapeutic development is emerging. Molecular targeted treatments have being combined with conventional anticancer drugs, accordingly with specific tumor biology.

Coming back to loco regional treatment of rectal cancer, IMRT might provide anatomical specificity. Molecular therapies will complement anatomical specificity by targeting biological pathways that are deregulated in individual tumors. Precision is technologically based while accuracy is biologically based[64].

New biological agents: biological modulation

Epidermal growth factor receptor (EGFR) and angiogenesis-related pathways are perhaps the molecular mechanisms best explored in colorectal cancer. Both mechanisms are involved either in colorectal carcinogenesis and tumor growth[65,66], and in radioresistence[67-69]. Thus, novel targeted biologic agents including angiogenesis and EGFR inhibitors hold tremendous promise as RT sensitizers and as systemic therapy in rectal cancer[69-71].

Preliminary reports show feasibility and promising activity combining Bevacizumab with 5-FU and RT. The MTD was determined for Bevacizumad at 5 mg/kg[72]. Additionally, surrogate markers are being investigated suggesting the ability of Bevacizumab to specifically target tumor angiogenesis[72,73].

A recent phase I study combining capecitabine, oxaliplatin and bevacizumab with preoperative RT establishes the MTD to be capecitabine 625 mg/m^2 BID, Oxaliplatin 50 mg/m^2 per week and Bevacizumab 15 mg/kg d 1 and 10 mg/kg d 8 and 22. Down staging was observed in 9/11 patients (82%) and 2/11 (18%) patients achieved pCR and in 2 of 11 only microscopic disease was found in the surgical specimen[74].

C225 (Cetuximab) is a chimeric monoclonal antibody that targets the extracellular domain of epidermal growth

factor receptor (EGFR) with high specificity and affinity[75]. Cetuximab has demonstrated increased responses combined with chemotherapy in metastasic colorectal cancer[76]. The radiosensitization activity of Cetuximab has been broadly explored[77]. Thus, the combination of chemotherapy and RT with C225 is an attractive strategy to be explored.

A pilot study has explored the addition of Cetuximab (250 mg/m^2 per week) to conventional i.v., continuous infusion of 5-FU and RT. Grade 3-4 diarrhea was detected in 10% and acneiform rash in 15%. Pathological complete response was achieved in 12% of patients[78].

Cetuximab has been combined with Capecitabine and RT in rectal cancer. The dose suggested is Capecitabine 825 mg/m^2 bid without interruption during the duration of RT and Cetuximab 250 mg/m^2 weekly. Grade 3 diarrhea was 10%, rectal pain 20%. Ten percent of the evaluated patients achieved pCR[79].

A phase I trail has recently evaluated the combination of Capecitabine, Oxaliplatin and C225 with RT. Doses suggested were for Cetuximab 400 mg/m^2 on d-7, then 6 weekly doses of 250 mg/m^2, for oxaliplatin 50 mg/m^2 d 1, 8, 22 and 29 in combination with capecitabine 1650 mg/m^2 bid d 1-14 and 22-35. Grade 3-4 diarrhea was 15% and grade 3-4 toxicity as skin reaction 7%[80]. The results of the phase Ⅱ study with 31 patients enrolled are coming soon.

Intensity Modulated Radiotherapy in rectal cancer: Rational and preliminary experience

New drugs and biological treatments may enhance global radiotherapy effects improving therapeutic outcomes but acute effects may also be increased. Moreover, a dose-volume relationship has been established between the severity of diarrhea toxicity and the volume of irradiated small bowel at all dose levels in patients treated with preoperative chemoradiation for rectal cancer[81]. The volume of irradiated small bowel thresholds to predict acute gastrointestinal toxicity is unknown although a strong correlation exists between the volume of small bowel receiving 15 Gy (V15) and the degree of acute small bowel toxicity[82].

The development of novel and sophisticated irradiation techniques as intensity modulated radiation therapy (IMRT) represents a spectacular progress in planning and delivering external beam radiation therapy. IMRT generates highly conformal and irregularly shaped dose distribution while reducing dose to adjacent normal tissue structures. IMRT has demonstrated dosimetric superiority over 3D-conformal radiation therapy (3D-CRT) in the majority of tumor sites, including pelvic tumors where the irradiated bowel can be significantly reduced[83].

Researchers at the Royal Marsden Hospital have reported a dosimetric study comparing IMRT *vs* 3D-CRT in five rectal cancer patients. The irradiated bowel volume at 45 Gy and 50 Gy can be reduced with IMRT techniques, which could potentially resulted in marked reductions in acute and chronic bowel toxicity[84]. Tho and colleagues[81] evaluated the role of IMRT in 41 patients with locally advanced rectal cancer treated with preoperative 5FU CHRT. The results showed that IMRT provided dosimetric and radiobiological modeling benefits by reducing the dose to the small bowel, and the likelihood of late normal tissue complications. A dosimetric comparison of 3D-CRT using pelvic anatomical references, 3D-CRT with more restrictive volumes, and IMRT was explored by our institution in nine patients diagnosed with locally advanced rectal cancer. A number of parameters, such as conformity index in the planning target volume, different dose levels at the planning target volume and organs at risk were calculated and compared between the three plans. Target coverage was similar, but the conformity index was better using IMRT. Irradiation doses at small bowel and bladder were significantly reduced with IMRT planning.

Dosimetric parameters in rectal cancer with IMRT are encouraging. Clinical research looking for acute and late toxicity, tumor response, tumor control and survival is warranted. The rationale for the use of chemo-IMRT in locally advanced rectal cancer is based on the potential decrease of gastrointestinal toxicity while maintaining conventional dose to the primary tumor, draining lymph node regions and presacral region. This capacity to change the gastrointestinal toxicity profile may also allow reducing the number of fractions by increasing fraction size, which ultimately may improve the rate of pCR and cost-effectiveness.

Our institution has carried out a prospective study of preoperative chemo-IMRT in rectal cancer. The treatment protocol includes simultaneous combination of capecitabine and oxaliplatin with three escalating dose levels of IMRT, 37.5 Gy 42.5 Gy and 47.5 Gy in 15, 17 and 19 fractions, respectively[85] Chemotherapy consisted on capecitabine 825 mg/m^2 bid during radiation therapy (resting over the weekend) and oxaliplatin 60 mg/m^2 d 1, 8 and 15. Resection was scheduled 6 wk after termination of chemo-IMRT. Simulation was made with the patient positioned prone and immobilized using a combination of prone head cushion and shell with a mixed foam bag. The patient was CT scanned from the L2 vertebral body to the entire perineum with a slice thickness of 5 mm. The slices were transferred through local network to the treatment planning system. The target volumes and organs at risk (OARs) were delineated on axial CT slices in the Helax-TMS treatment planning system (Nucletron Scandinavia, Uppsala, Sweden) as seen in Figure 1. The gross tumor volume (GTV) was defined as the primary tumor and the suspicious metastasic lymph nodes visualized on the CT scan. The clinical target volume (CTV) included the GTV, the presacral region and the common and internal iliac lymph nodes. Adding a margin of 0.5-1 cm around the CTV generated the planning target volume (PTV). The OARs outlined were the bladder and the small bowel. After the GTV, CTV, PTV and OARs were contoured the edited CT slices were transferred from the Helax-TMS treatment planning system to the inverse planning system (KonRad version 2, Siemens Oncology Care Systems, Heidelberg, Germany). Inverse planning for step-and-shoot treatment was performed using 15 MV photons generated on a Mevatron Primus linear accelerator (Siemens Oncology Care Systems, Concord, USA). Seven coplanar equally spaced fields (gantry angles 0°, 51°, 103°,

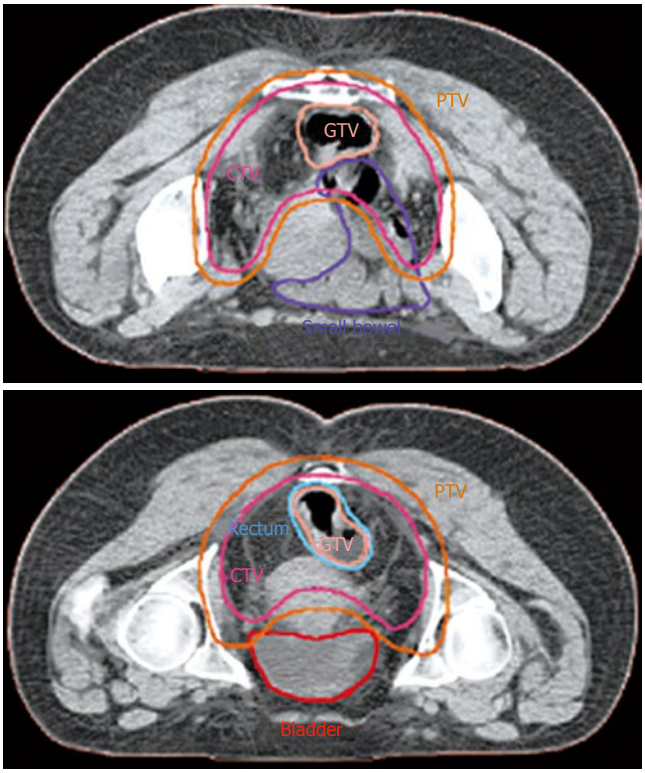

Figure 1 The GTV, PTV and organ at risk (small bowel and bladder) countered on the axial CT slices.

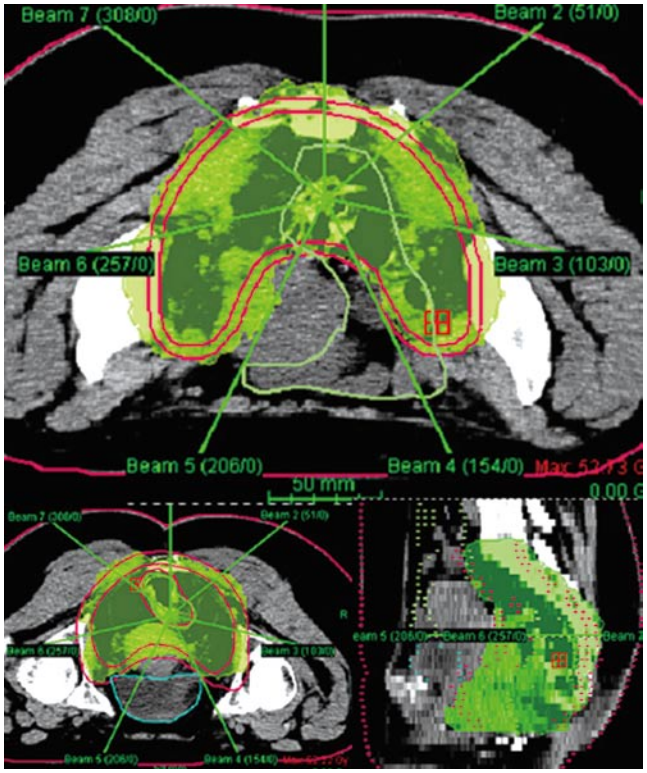

Figure 2 Axial and sagital CT scan images with dose distributions. The 45 Gy isodose surface (green) encompass the GTV and PTV.

154°, 206°, 257° and 308°) were used and the isocenter was placed in the geometric center of the PTV. Figure 2 displays the clinical dosimetry over the patient CT scans.

The first three patients received 37.5 Gy and there were no dose-limiting toxicity (DLT) defined as any grade 3 or 4 gastrointestinal toxicities or grade 4 hematological toxicity. The next three patients received 42.5 Gy without observed DLT and the remaining patients received 47.5 Gy in 19 fractions. Preliminary data show that treatment compliance was 80%, grade 3 adverse events were seen in 21% of the cases, down staging was observed in 52% of patients and pathological response grade 3+ or 4 according to the scale established by Ruo *et al*[86] occurred in 45% of patients.

The use of preoperative IMRT combined with more active systemic chemotherapy provides a major challenge to improve treatment-related toxicity observed with more conventional radiation techniques. Furthermore, the promising favorable pathological response observed with these strategies has the potential to be associated with better loco regional control of disease and may predict better survival.

CONCLUSIONS

Preoperative CHRT followed by TME surgery is the current framework for rectal cancer treatment picture. Further advances with better agents (chemotherapy and molecular targeted therapies) and technology (IMRT) will be translated to improved shapes and colors, enhanced contrast and brightness: response intensity with balanced toxicity.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Moving forward in colorectal cancer research, what proteomics has to tell

Nerea Bitarte, Eva Bandrés, Ruth Zárate, Natalia Ramirez, Jesus Garcia-Foncillas

Nerea Bitarte, Eva Bandrés, Ruth Zárate, Natalia Ramirez, Jesus Garcia-Foncillas, Laboratory of Pharmacogenomics, Center for Medical Applied Research, Unit of Clinical Genetics, University Clinic, Department of Oncology and Radiotherapy, University Clinic, University of Navarra, Pamplona 31008, Spain
Correspondence to: Jesús García-Foncillas, MD, PhD, Laboratory of Pharmacogenomics, Center for Medical Applied Research, Department of Oncology and Radiotherapy, University Clinic, University of Navarra, Avda Pio XII 36, Pamplona 31008, Spain. jgfoncillas@unav.es
Telephone: +34-948-194700-1008 Fax: +34-948-194718
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Abstract

Colorectal cancer is the third most common cancer and is highly fatal. During the last several years, research has been primarily based on the study of expression profiles using microarray technology. But now, investigators are putting into practice proteomic analyses of cancer tissues and cells to identify new diagnostic or therapeutic biomarkers for this cancer. Because the proteome reflects the state of a cell, tissue or organism more accurately, much is expected from proteomics to yield better tumor markers for disease diagnosis and therapy monitoring. This review summarizes the most relevant applications of proteomics the biomarker discovery for colorectal cancer.

Key words: Proteomics; Colorectal cancer; Biomarker

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INTRODUCTION

Cancer is not a single disease, but an accumulation of genetic and epigenetic events. It is characterized by uncontrolled growth of cells that can invade and destroy normal tissues. These abnormal cells can also spread through the bloodstream or lymph system to start new tumors in other parts of the body. The disease is a great challenge to clinicians and scientists.

Recent progress in molecular biology has allowed the identification of markers useful for patient management through the identification of genetic alterations and an understanding of chemotherapy molecular targets. Several examples in digestive oncology underline the relevance of molecular biology in clinical research[1].

Colorectal cancer is a common malignancy with an annual incidence of over 945 000 cases worldwide and an annual mortality of 492 000[2]. Surgery is the treatment of choice offering a potential cure. However, 30%-40% of patients have local regionally advanced or metastatic disease on presentation, which cannot be cured by surgery alone[3]. In addition, more than half of patients initially believed to be cured develop recurrence and die of the disease[4].

Advances in genomics and proteomics contribute to our understanding of pathways that control growth, differentiation, and death of cells. In these processes, the identification of candidate disease genes and modifier genes by integrated study of gene expression and metabolite levels is instrumental for future health care. This approach, called systems biology, can recognize early onset of disease and identify new molecular targets for novel drugs in cancer[5].

Proteomics analyzes proteins within a cell or in the corresponding tissue; the proteins of interest are identified, but their function and interactions are not determined. The research provides complete and detailed data about structure, expression, and function of genes, but fails to demonstrate how all the information implicated in the genome is used. In the "post-genomic era," proteomics might be the key to understand systems biology. During the past few years, proteomics has been utilized in many fields of science, medicine, pharmacy, industry and agriculture[6]. In most of the applications proteomics is used to determine expression profiles of proteins in cells and tissues in normal or disease states[7] that are responsible for abnormal cell proliferation. The identification of proteins that are characteristic for cancer development can potentially uncover diagnostic, or prognostic markers, or novel drug targets, and could help understand the mechanisms underlying tumor formation (Figure 1).

Currently, proteomic technology has been used in two areas of cancer research, in early diagnosis and in the treatment of patients, that also includes prediction of response. This technology, when combined with

genomic analysis, may provide more information about the molecular basis of carcinogenesis and the development of more effective anti-cancer therapies. This review focuses on the proteomic studies applied in colorectal cancer.

PROTEOMIC TECHNIQUES IN CANCER RESEARCH

Sample preparation in proteomic

Sample preparation is the most critical step in any proteomics study. This is important because it affects reproducibility as a result of the heterogeneity of proteins derived from cell populations[8]. From the time of sample collection to when proteins are processed for analysis, multiple factors come into play. Mechanical methods, such as surface scrapping and fine needle aspiration, have been used for capturing cancer cells[9]. Calcium depletion and other nonenzymatic methods, such as immunomagnetic separation, have been used to obtain pure populations of cancer cells[10]. An important advancement in sample preparation has been the development of laser capture microdissection (LCM). The LCM system permits obtaining pure populations of cancer cells from frozen, paraffin-embedded, stained, and unstained tissues for molecular analysis. The system is based on visualizing a tissue section via light microscopy and procurement of cells by activating a 7.5-30 micron diameter infrared laser beam which adheres the tissue to a plastic cap. Intact deoxyribonucleic acid, RNA, and protein are then extracted from the adhered tissue which then can be analyzed using conventional methods[11,12]. Protein expression has been compared using 2-D PAGE and differentially expressed proteins identified by mass spectrometry, permitting the discovery of a novel colorectal cancer biomarker[13,14].

Two-dimensional gel electrophoresis and tumor protein detection (2D)

Traditional proteomic studies are based on 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to compare protein expression patterns from different tissues or cell lines. The first dimension separates proteins by pH, isoelectric focusing, and the second dimension by molecular mass, sodium dodecyl sulfate PAGE. Although, 2-D PAGE has been available for several decades, improvements in this technology have dramatically improved sensitivity, resolution and reproducibility.

The more important application of this technique in disease proteomics is the discovery of proteins which might serve as prognostic biomarkers for survival of cancer patients. A novel application of 2-D PAGE has been in the discovery of circulating autoantibodies in cancer patients. In some cancer patients, there is evidence that a humoral immune response against tumor antigens might be elicited, and this might be used in serum assays of disease progression or in the development of anticancer vaccines.

An advantage of 2-D PAGE is that it has the capacity to resolve and investigate protein, abundance in a single sample and the possibility to directly detect changes in diseased and healthy tissue.

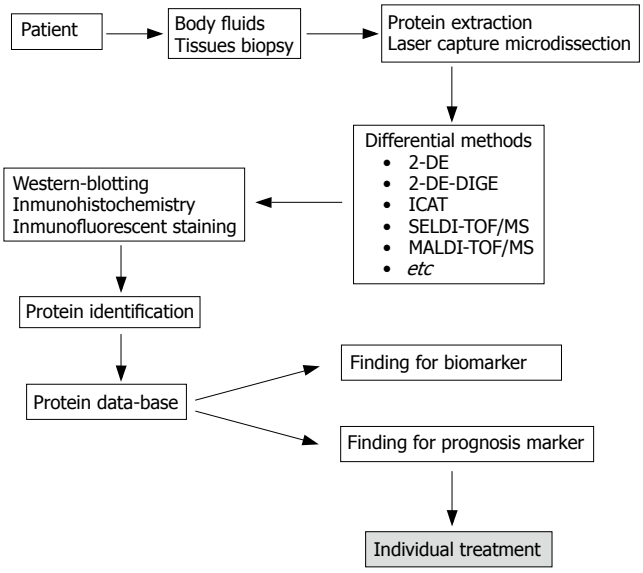

Figure 1 Proteomic differential display methods.

The major disadvantage of 2-D PAGE is that it is laborious and does not resolve highly basic or proteins, smaller than 10 kDa. Because most clinical biomarkers are high large proteins 2-D PAGE is an ideal technology for the study of cancer biomarkers. Therefore, 2-D PAGE, complemented with mass spectrometry, has been used to identify protein changes associated with a variety of human cancers[12].

Two-dimensional difference gel electrophoresis (2D-DIGE)

One of the most recent technical advances in 2-DGE has been multiplexing fluorescent 2D-DIGE[15]. This method directly labels lysine groups in proteins with cyanine (Cy) dyes prior to IEF and can allow for quantitative comparisons between patients and control samples when different fluorescent labels are used for each sample.

The critical aspect of 2D-DIGE technology is the ability to label 2-3 samples with different dyes and then electrophorese all samples on the same 2-D gel. This ability reduces spot pattern variability and the number of gels in an experiment making spot matching much more simple and accurate[16]. The single positive charge of the CyDye replaces the single positive charge present in the lysine at neutral and acidic pH keeping the pI of the protein relatively unchanged. A mass of approximately 500 Da is also added by the CyDye to the labeled protein. The individual protein data from the control and diseased/treatment (Cy5 or Cy3) samples are normalized against the Cy2 dye-labeled sample, Cy5:Cy2 and Cy3:Cy2. These logarithm abundance ratios are then compared between the control and diseased/treatment samples from all the gels using statistical analysis (*t*-test and ANOVA)[17,18]. The principal disadvantage of this technique is that it has a low throughput (three samples per gel) (Figure 2).

Antibody, protein and peptide arrays

Antibody array based measurement technologies have long provided an important tool to detect and manipulate specific biological molecules. While previous uses of

antibodies and related affinity reagents have focused on single targets, recent developments have included multiplexed use of antibodies in arrays, so that many targets can be measured in parallel, sometimes in very small sample volumes. The uses of such arrays are varied and new applications and formats continue to evolve[19].

The experimental features of microarrays have advantages for cancer research. The low sample volumes result in the consumption of small amounts of both precious clinical samples and expensive antibodies. The assays can be run efficiently in parallel, making possible studies on the large populations of samples that are necessary for marker detection and validation. In addition, these assays have good reproducibility, high sensitivity, and quantitative accuracy over large concentration ranges[20]. Antibody and protein arrays are complementary and in some aspects preferable to separation based and mass spectrometry based technologies. Reproducibility and throughput can be higher, and the identities of the considered proteins are known or can be readily characterized. Therefore, specific hypotheses regarding the nature of molecular alterations can be tested, and biologically interpreted[21]. Applications of antibody array methods to cancer research are increasing in scale and effectiveness.

Protein and peptide arrays are effective for probing the interactions of protein and peptides with other antibodies, protein or other molecules. Protein microarrays are an emerging class of nanotechnology for analysing many different proteins simultaneously. Much progress has been made for applications in basic science[22]. These approaches are likely to recapitulate at the protein level the mRNA expression profiling studies by arraying various protein probes on top of specific surfaces, and then determining interactions with specific proteins in complex samples. The most advanced format in this setting is the antibody microarray, where the proteins are specific antibodies printed on solid surfaces.

Protein arrays recently have confirmed the use for probing the abundance of specific proteins in biological samples, this phase call "reverse phase". Protein lysates from cell culture or tissue samples are spotted in microarrays on nitrocellulose membranes. A labeled antibody specific for a particular protein is incubated on a microarray, and quantification of the bound antibody reveals the amount of that protein in each sample[23,24]. Therefore, reverse phase array experiments quantify a single protein in many samples, in contrast to antibody arrays that quantify many proteins in one sample. Numerous demonstrations that this technology uses for profiling proteins in cancer have appeared.

The various methods presented here are complementary with each other and with other proteomic methods, and they may be used together for added benefit as demonstrated in a study of proteins in breast cancer cells using cytokine arrays, reverse phase arrays, and bead-based arrays in conjunction with two-dimensional gels (Figure 3).

TOF-Mass Spectrometry applications in clinical oncology

SELDI-TOF MS is a commonly used non-gel based method. The technique combines protein separation directly with presentation to the mass spectrometer. Various types

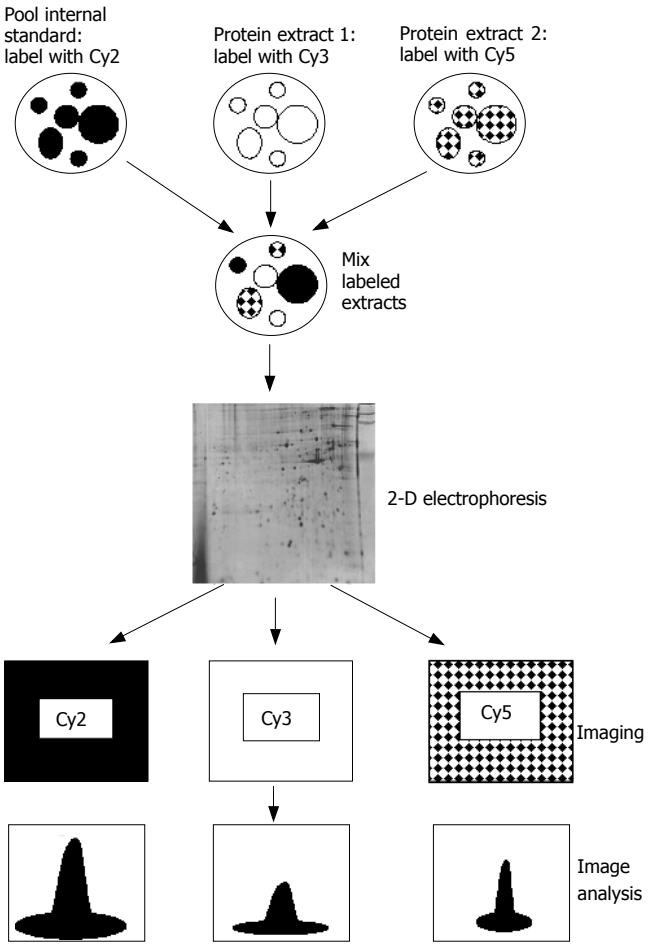

Figure 2 2D-DIGE techniques. Cy2, Cy3 and Cy5 are different fluorescent dyes.

of substrates have different affinities for different proteins, thus it is possible to increase protein representation when combining various arrays. The combination of these arrays with up-front prefractionation chromatography (e.g., anion exchange) permits the detection of up to 2000 protein species from serum[25,26]. The resulting spectral masses are analyzed using univariate and multivariate statistical instruments to provide a single marker or multimarker pattern that can classify clinical samples. Discriminator protein pinnacles are then purified and submitted to the MSbased identification process (Figure 4).

The SELDI technique was developed to profile clinical biological fluids, notably serum and/or plasma, and became important when numerous studies showed its potential in identifying unique biomarkers or complex patterns with diagnostic value, allowing its use for screening and early diagnosis in various cancers[27,28]. One major criticism of the technique relies on the overall lack of sensitivity and capability to detect tumor-specific protein traces within a large amount of nonspecific protein species[29]. However, even though still controversial in its reproducibility and ability to detect actual specific tumor signatures, SELDI has several advantages, such as easy of use, high throughput, and relatively reasonable cost, all making it a very attractive technique for working with large clinical sample.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), is a

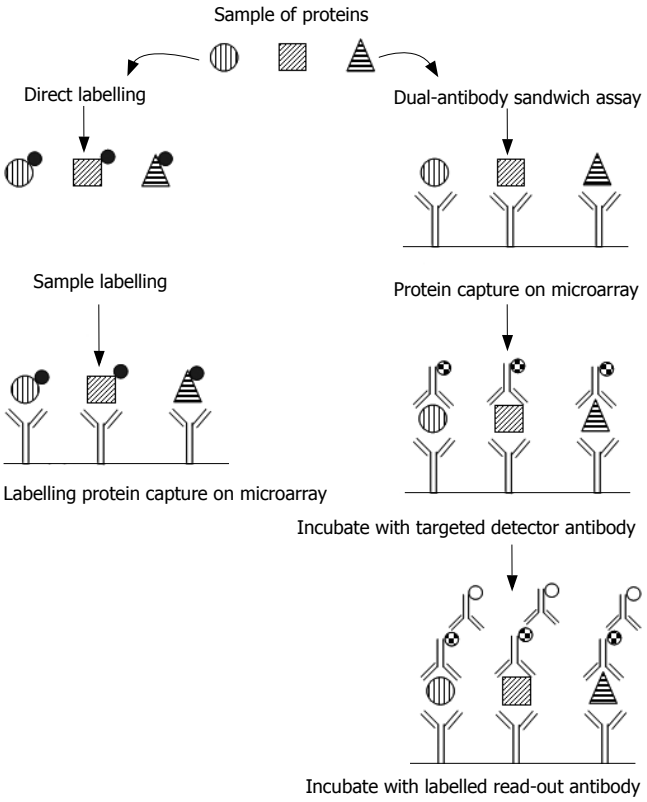

Figure 3 Representation of the two antibody microarray experimental formats. Direct labelling: single-capture antibody experiments; all proteins in a sample are labelled (black circles) thereby providing a means for detecting bound proteins following incubation on an antibody microarray. Dual-antibody (capture and read-out antibody) sandwich immunoassays: proteins captured on an antibody microarray are detected by a cocktail of tagged detection antibodies, which are matched to the spotted antibodies. The detector antibody tag is then measured by binding of a labelled (empty circles) read-out antibody.

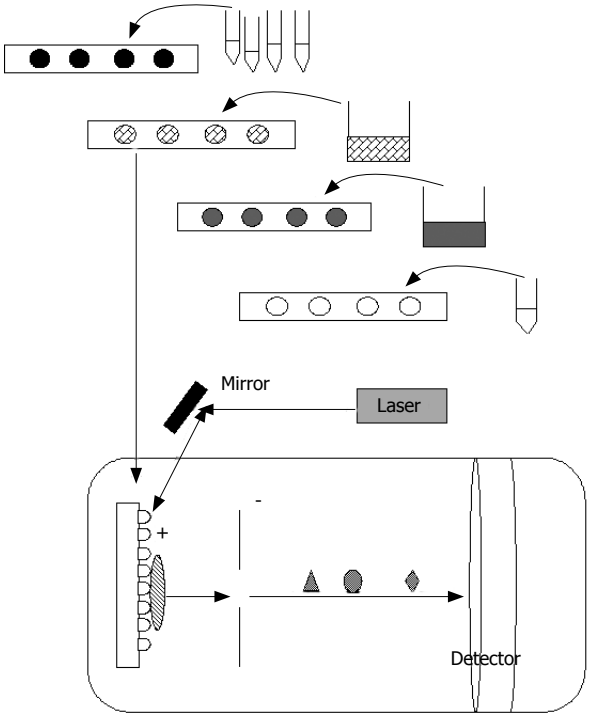

Figure 4 Principles of SELDI-TOF MS. The application of sample from to an eight-spot array with hydrophilic, hydrophobic, cationic, anionic or immobilized-metal affinity capture chromatography surface (black colour). The addition of an appropriate binding buffer (purple colour). On-chip sample purification using one or more wash buffers (grey colour). The application of energy-absorbing matrix for the absorption of laser energy (empty colour). Laser irradiation desorbs bound proteins and positively ionizes them. Owing to the electric field, they migrate in the mass analyser: (small diamond) and multiply charged proteins (oval) faster than large and single-charged ones (triangle). Thus, the proteins are separated. Time of flight (t) is proportional to protein mass per charge.

technique to analyze peptides and proteins in relatively complex samples. It has even been used for the direct analysis of tissue specimens[30]. In MALDI-TOF MS, a small quantity of specimen containing peptides and protein is dried on a target plate together with a light-absorbing matrix molecule.

Two technical advancements have improved resolution of MALDI-TOF MS to its current state. First, use of an electronic mirror (reflectron) to reflect ions substantially increases resolution, and second, delayed extraction introduced after sample vaporization and earlier than the electric potential is applied. Shorter times are optimal for small molecules, and longer times for large molecules. The standard detector for MALDI-TOF MS is a microchannel plate, which acts as an electron multiplier for ions reaching the detector. Detector replys relate to the number of ions reaching the detector and ion velocities.

MALDI-TOF MS permits a rapid determination of molecular masses and the heterogeneity of small amounts of peptides and proteins. Usually, intact molecular ions are formed and determination of polypeptide mass.

LC-MS and LC-MS-MS in comparative proteomic

Capillary-scale HPLC-MS/MS (LC-MS) is rapidly emerging as a method of choice for large scale proteomic analysis[31]. LC-MS systems can be used to identify and track the relative abundance of thousands of molecules[32]. For standard bottom-up profiling experiments, the molecules in question are peptides derived by proteolysis of intact proteins. For very complex protein samples, such as blood, the peptide mixtures are resolved by chromatographic separation prior to injection into the mass spectrometer. This generates a more informative map, that consists of both the unique elution of individual peptides. Distinct peptides of interest are induced by collision fragmentation followed by database matching for the purpose of sequence identification, while the recorded pattern of precursor ion intensities can be used to infer the relative quantities of the various proteins between samples[33].

LC-MS systems consists of different instruments to separate peptide mixtures based on physicochemical properties, separate ions on the basis of m/z ratios and registers the relative abundance of ions at discrete m/z.

In LC-MS-MS technique, precursor ions are recorded in full-scan mode, followed by selective ion isolation and fragmentation for sequence identification[33] (Figure 5).

Isotope-coded affinity tags (ICAT and iTRAQ)

This is the prototypical and the most popular method for quantitative proteome analysis based on stable isotope affinity tagging and MS[34].

The ICAT reagent is a sulphydryl-directed alkylating agent composed of iodoacetate attached to biotin through

a short oligomeric coupling arm (d0). The exchange of 8 deuterium atoms for hydrogen atoms in the coupling arm produces a heavy isotope version of the reagent (d8). Thus the reagent comprises of a cysteine reactive group, a linker containing the heavy or light isotopes (d8/d0) and a biotin affinity tag. This method involves *in vitro* derivatization of cysteine residues in protein with d0 or d8 followed by enzymatic digestion of the combined sample. All the cysteine residues thus tagged with biotin are selectively separated by avidin column and the cysteine-containing peptides are further separated followed by MS analysis[35].

The iTRAQ technique capable of multiplexing samples is primarily based on the ICAT technique and compared in detail. The iTRAQ technique uses four isobaric reagents allowing the multiplexing of four different simples in a single LC-MS-MS experiment. The multiplexing capability of iTRAQ allows a control sample to be compared with different points in time of a disease state, as well as with respect to different drug treatments. One of the major advantages of this technique is its ability to label multiple peptides per protein, which increases the confidence of identification and quantitation[16].

There are numerous differences (advantages and disadvantages) between the select proteomic technologies for protein profiling (Table 1).

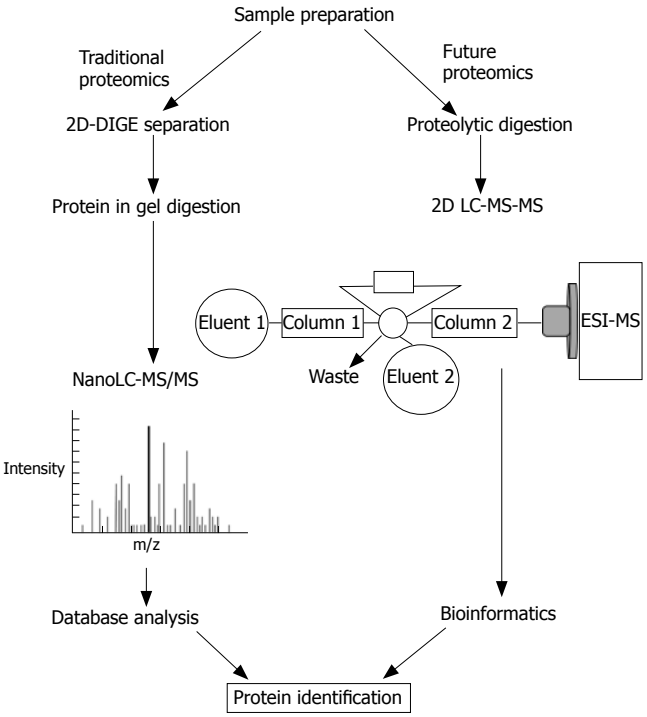

Figure 5 Different strategies for proteomic studies.

High-resolution hybrid quadrupole TOF

One of the first major advances used in any developing area of research was a high-resolution hybrid quadrupole TOF (QqTOF) MS fitted with a SELDI ion source to acquire proteomic patterns from serum. A recent study was designed to determine whether there is any diagnostic advantage provided by acquiring the proteomic patterns of serum samples using a high-resolution, high mass accuracy MS instrument. Results were analyzed on the exact same ProteinChip surface, thus eliminating all experimental variability apart from the use of two different instruments. Different combinations of bioinformatic heuristic parameters were used to generate different diagnostic models using the data acquired from the two distinct mass spectrometers[35]. These parameters included the similarity space for cluster classification, and the learning rate in training of the genetic algorithm. The diagnostic models generated from mass spectra acquired using the higher-resolution Qq-TOF MS were statistically superior[36].

Proteomic analysis software

The result of the analysis of a complex proteomic mixture by SELDI-TOF-MS is a low resolution profile of the protein or peptide species that were subsequently ionized from ProteinChip surface. It has been the development and combination of sophisticated bioinformatic algorithms for the analysis of SELDI-TOF-MS data. The intention of this bioinformatic analysis has led to the potential application of this technology as a major advancement in the diagnosis of cancer and other diseases. There are several different types of bioinformatic algorithms, such as single classification trees, neural nets, genetic algorithms, and random forest algorithms, which have been applied to enable SELDI-TOF-MS data to be investigated as a diagnostic technology. Although they function in different protocols, these algorithms share a common goal: to construct a classifier and discover peak intensities most likely to be responsible for segregating classes of samples[37]. Since its inception, SELDI-TOF-MS has been used to develop diagnostic platforms for several different cancers.

PROTEOMIC ANALYSIS IN COLORECTAL CANCER

During the past decade, genomic analyses have been introduced into cancer studies with variable success. It has become recognized that genomic techniques are insufficient to study the complex pathways of carcinogenesis; this has led to the application of proteomic techniques, which allow for the reliable analysis of complex mixtures of proteins[38].

Colorectal cancer is the third most common cancer in the world. It is well known that the adenomatous polyposis coli (APC) gene is mutated in patients with familial adenomatous polyposis (FAP) and sporadic colorectal cancer, and that mutations initiate colorectal carcinogenesis. It is now suggested that many colorectal cancers arise from preexisting adenomas. Following several steps of mutation of oncogenes and tumor suppressor genes, adenomas develop to colorectal cancers[7].

Many groups have reported the proteomic analyses of colorectal cancers. Dundas *et al*[39] found that mortalin, also known as mitochondrial HSP70, is involved in cell cycle regulation with important roles in cellular senescence and immortalization pathways and was over-expressed in colorectal adenocarcinomas and correlated with poor survival. Lane *et al*[40] identified over-expressed multiple cytochrome P450 enzymes in human colorectal cancer tissues and metastases. Cytochrome P450 proteins (CYPs)

Table 1 Advantages and disadvantages of proteomic technologies for protein profiling

Technique	Methods	Advantages	Disadvantages
2D	Separation on a gel of the protein content of a sample in two dimensions according to mass and charge; gels are stained and spot intensities in samples are compared among different gels	High separation (thousands of proteins per gel)	Low throughput laborious (one samples per gel); poor resolution for extreme masses and extremely acidic or basic proteins; no direct protein identification; large amount of starting material compared with other techniques
2D-DIGE	Measuring three samples per gel; each of them is labelled with a different fluorescent dye, and the intensities of each gel spot for each sample are measured at a wavelength specific for the label	Direct comparison of samples on one gel: better reproductibility	Low throughput (three samples per gel)
Protein microarrays	Binding of a targeted protein in one sample to spotted probes on a 'forward' microarray; conversely, binding of specific probes to a targeted protein in spotted samples on a 'reverse' microarray; detection of bound proteins by direct labelling or by labelled secondary antibodies	High throughput in terms of number of probes per (forward) array or number of samples per (reverse) array; biomarker identity or class readily known	Synthesis of many different probes necessary; identity or class of targeted proteins must be known; limited to detection of proteins targeted by the probes
SELDI-TOF MS	Selected part of a protein mixture is bound to a specific chromatographic surface and the rest washed away	High throughput; direct application of whole sample (fast on-chip sample cleanup); small amount of starting material	Unsuitable for high molecular weight proteins; limited to detection of bound proteins; lower resolution and mass accuracy than MALDI-TOF
MALDI-TOF MS	Application of a protein mixture onto a gold plate; desorption of proteins from the plate by laser energy and measurement of the protein masses; comparison of peak intensities between multiple samples	High throughput	Need for sample fractionation of complex samples; more starting material needed for sample fractionation; unsuitable for high molecular weight proteins
LC-MS-MS	Separation of a mixture of peptides (resulting from protein digestion with trypsin) by one-, two-or three-dimensional LC and measurement of peptide masses by MS-MS	Direct identification of several hundred proteins per sample by MS-MS of peptides	Low throughput; time consuming; detection by MS–MS often not comprehensive, tus complicating comparison of different samples
ICAT	Chemical tagging of proteins on cysteine residues with a heavy or light stable isotopic; after labelling samples are mixed, proteins are digested with trypsin, and labelled peptides isolated by affinity chromatography; both samples are analysed concomitantly by LC-MS-MS	Direct identification of biomarkers by MS-MS of peptides; relative quantitation; less sample complexity than with iTRAQe; MS-MS of only differentially expressed proteins	Low throughput; tagging of only cysteine-containing peptides
iTRAQ	Chemical tagging of proteins on their amine groups with stable isotopic labels of identical mass ('isobaric'); four different labels are available for four different samples; after labelling, samples are mixed, proteins digested with trypsin and analysed concomitantly by LC-MS-MS	Direct identification of biomarkers by MS-MS of peptides; owing to isobaric labels, selection for MS-MS of the same peptide in all four samples in the same single MS run	Low throughput (four samples per run); for generating signature ion, MS-MS of all peptides in a sample is necessary; high sample complexity and limited resolution of LC (even three dimensional), confounding by co-eluting isobaric peptides

in the liver are known to be of major importance to the fate of anticancer agents; however, their expression and role in tumours has received little attention. CYP-mediated metabolism is generally viewed as a route to drug detoxification and increased elimination, although CYP activation of certain anticancer drugs. The presence of metabolically active CYPs in a colon metastastic deposit is likely to be important in determining the metabolic fate of chemotherapeutic agents and hence the outcome of treatment. Stulik *et al* performed proteomic differential display between the matched sets of macroscopically normal colon mucosa and colorectal cancer tissues. They report that the expression of HSP70, S100A9, S100A8, S100A11 and S100A6 was up-regulated in colorectal cancer tissues compared to normal colon mucosa, and the levels of liver fatty acid-binding protein, actin-binding protein/smooth muscle protein 22-a and cyclooxygenase 2 were down-regulated in transformed colon mucosa[41]. The S100A6 protein was the first S-100 protein specifically identified as being related to the state of cellular proliferation. The possible correlation between increased expression of some members of the S100 protein

Table 2 Proteomic analysis in human colorectal cancer tissues

Up-regulated		Down-regulated
Annexin Ⅳ		NCF2
MTA-1		PMM2
SSX5 protein		Serpin 1
Dynein heavy chain		CNRC
Cytochrome P450		Annexin Ⅴ
CPT1		APC
Keratin 10		VAV3 protein
Keratin 8		RSP 4
Keratin 19		SPARC like protein 1
Vimentin		PDI
β-actin		GN6ST
REL1		Cathepsin D
HSP60		Calreticulin
Mortalin	Cathepsin fragment	SM31
		PDA6
	Proteasome subunit a type 6	ApoA1 precursor
Cytochrome P450 enzymes		ATP synthase b chain
(in cancer tissues and metastatic tissues)	Triosephosphate isomerase 14-3-3 proteins	Albumin
		Liver fatty acid-binding protein
HSP70	GST-P	Actin-binding protein/smooth muscle protein 22-a
S100A9		
S100A8	P13693 translationally controlled tumor protein	Cyclooxygenase 2
S100A11		
S100A6		Puromycin-sensitive aminopeptidase
	Nucleoside diphosphate kinase A	
		NADH-ubiquinone oxidoreductase
Adenosyl homocysteinase	Calgranulin B; S100 A9	
Leukocyte elastase inhibitor, claude B		Succinate dehydrogenase subunit A
Macrophage capping protein		
Biliverdin reductase A		Aldehyde dehydrogenase, cytosolic, class Ⅰ
Annexin 1 fragment		
α-tubulin		
Elongation factor 1-d		Selenium-binding protein
Tropomyosin a1		Creatin kinase B chain
Tropomyosin a4 chain		Placental thrombin inhibitor
Actin fragment		Vimentin
Annexin 5		Desmin
Microtuble-associated protein RP/EB		Tubulin b 5 chain
Pyridoxal kinase		Carbonic anhydrase Ⅰ
Annexin 3		Myosin regulatory light chain 2
Annexin 4		

family and colon carcinogenesis is also supported by the finding that documents the participation of the S100A4 protein in the progression and metastasis of colorectal carcinogenesis. Alfonso *et al*[42] reported the up-regulation of annexin Ⅳ, MTA-1 and others in colorectal cancer tissues, and the down-regulation of NCF2, PMM2 and others. Several functional groups of proteins were affected, including regulators of transcription, structural proteins, and those involved in protein synthesis and folding. The MTA-1 gene encodes a protein that was identified in metastatic cells, specifically, mammary adenocarcinoma cell lines. Expression of the MTA-1 gene has been associated with the progression of several carcinomas in colon, lung, prostate, and liver. A annexin Ⅳ is a calcium-binding protein and I involved in cellular communication and signal transduction, for this reason it was up-regulated in colorectal cancer. Friedman *et al*[43] identified adenosyl homocysteinase, leukocyte elastase inhibitor and others as up-regulated proteins, and puromycin-sensitive aminopeptidase, NADH-ubiquinone oxidoreductase and others as down-regulated proteins in colorectal cancer tissues.

Minowa *et al*[44] identified truncated β-tubulins as a protein specific to polyp samples from APC gene-mutant mice by proteomic analysis of the small intestine and colon epithelia. The adenomatous polyposis coli gene (APC) is mutated in patients with familial adenomatous popyposis (FAC) and sporadic colon cancer, and these mutations initiate colon carcinogenesis. Simpson *et al*[45] performed membrane proteomic analysis of the human colon carcinoma cell line LIM 1215 to search for novel tumor marker proteins expressed during various stages of cancer progression, although the data are not shown.

Given the continual rise in the number of potential biomarkers of CRC, future studies will increasingly employ genomic and proteomic technologies, which enable the measurement and analysis of numerous potential biomarkers simultaneously. These techniques are able to produce gene or protein 'profiles' associated with clinical outcome, the analysis of which may then yield novel biomarkers with prognostic and/or therapeutic potential[46] (Table 2).

At this moment, biomarkers whose sensitivity and

specificity are better than bloody stool examination have not yet been found. Since the bloody stool test is easier than examination using cancer specimens and easier to handle than sera, from a clinical aspect, the bloody stool examination is better than biomarkers[34].

In another recent study, the detection of upregulated α-defensins 1, 2 and 3 in colorectal cancer tissue were reported in two independent, but similar analyzes. In both studies, SELDI-TOF MS results in tissue correlated with serum levels that were determined using ELISA or SELDI-TOF MS. This provides an interesting approach for finding new serum markers because biomarkers identified first in tissue could prove to be more specific. Unfortunately, α-defensin levels are also increased in serum during, for example, infection[47]. α-defensin and β-defensin are major components of the epithelial mammalian innate immune system. Defensins are small cationic peptides with high activity against a variety of microbials, encoded by genes and some are regulated in response to challenge with bacterial antigens. Gastrointestinal α-defensins (HD5 and HD6) are almost exclusively expressed in and secreted from Paneth cells of the small intestine, while β-defensins (hBD-1, hBD-2, hBD-3) are secreted by virtually all gastrointestinal epithelial cells to a varying extent.

CONCLUSIONS AND FUTURE PERSPECTIVES

Rapidly developing techniques that considerably enhanced information gained from proteomes integrate proteomics with other disciplines such as cell biology, biochemistry, molecular genetics, and chemistry. This consolidation certainly demonstrates incredible power and possibilities of proteomics for further applications. It is necessary to cross the barriers of limited resolution, mass range, detection level, and other reasons for protein underrepresentation in analyzed proteomes. Once achieved, the door that allows complete identification of specific protein markers will open and the comprehension of complex networks of protein/peptide interactions involved in cancer will begin to be elucidated[6]. While the application of computational and statistical methods to proteomic profiling is relatively new, it is rapidly gaining interest. Hence, it is worthwhile suggesting fruitful avenues for moving forward. It was suggested above that simultaneous LC-MS data alignment and normalization may be beneficial for comparative profiling.

Proteomic technologies are now in place to examine simultaneously and comprehensively many protein expression differences that result from disease and treatment, with the ultimate payoff being the use of specific protein profiles for the early diagnosis of patients and for patient-tailored therapies[47].

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Immunotherapy and immunoescape in colorectal cancer

Guillermo Mazzolini, Oihana Murillo, Catalina Atorrasagasti, Juan Dubrot, Iñigo Tirapu, Miguel Rizzo, Ainhoa Arina, Carlos Alfaro, Arantza Azpilicueta, Carmen Berasain, José L Perez-Gracia, Alvaro Gonzalez, Ignacio Melero

Guillermo Mazzolini, Catalina Atorrasagasti, Miguel Rizzo, Liver Unit, Facultad de Ciencias Biomédicas, Universidad Austral, Presidente Perón 1500, (1635) Derqui-Pilar, Buenos Aires, Argentina
Oihana Murillo, Juan Dubrot, Iñigo Tirapu, Ainhoa Arina, Carlos Alfaro, Arantza Azpilicueta, Carmen Berasain, José L Perez-Gracia, Alvaro Gonzalez, Ignacio Melero, Centro de Investigación Médica Aplicada and Clínica Universitaria, Universidad de Navarra, Pamplona, Spain
Correspondence to: Guillermo Mazzolini, MD, PhD, Liver Unit, Universidad Austral, Av Presidente Perón 1500, (1635) Derqui-Pilar, Buenos Aires, Argentina. gmazzoli@cas.austral.edu.ar
Telephone: +54-2322-482618
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Abstract

Immunotherapy encompasses a variety of interventions and techniques with the common goal of eliciting tumor cell destructive immune responses. Colorectal carcinoma often presents as metastatic disease that impedes curative surgery. Novel strategies such as active immunization with dendritic cells (DCs), gene transfer of cytokines into tumor cells or administration of immunostimulatory monoclonal antibodies (such as anti-CD137 or anti-CTLA-4) have been assessed in preclinical studies and are at an early clinical development stage. Importantly, there is accumulating evidence that chemotherapy and immunotherapy can be combined in the treatment of some cases with colorectal cancer, with synergistic potentiation as a result of antigens cross-presented by dendritic cells and/or elimination of competitor or suppressive T lymphocyte populations (regulatory T-cells). However, genetic and epigenetic unstable carcinoma cells frequently evolve mechanisms of immunoevasion that are the result of either loss of antigen presentation, or an active expression of immunosuppressive substances. Some of these actively immunosuppressive mechanisms are inducible by cytokines that signify the arrival of an effector immune response. For example, induction of 2, 3 indoleamine dioxygenase (IDO) by IFNγ in colorectal carcinoma cells. Combinational and balanced strategies fostering antigen presentation, T-cell costimulation and interference with immune regulatory mechanisms will probably take the stage in translational research in the treatment of colorectal carcinoma.

Key words: Colorectal carcinoma; Immunotherapy; Gene therapy; Interleukin-12; Dendritic cells; CD137; Indoleamine 2, 3 dioxygenase

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INTRODUCTION

Conventional therapy for cancer is based on surgical resection, chemotherapy with drugs with selective toxic effects against dividing cancer cells, and localized gamma irradiation. Biological therapy has only recently been introduced[1]. This includes the use of agents that interfere with growth factors for malignant cells, and block tumor neovascularization[2]. Among the monoclonal antibodies (mAbs) that have been approved for cancer treatment, most operate *via* indirect mechanisms, and only a minority target natural or artificial mechanisms of cell destruction.

Colorectal carcinoma (CRC) is one of the leading causes of cancer-related deaths worldwide[3]. Unfortunately, more than 20% of patients with CRC have metastatic disease at the time of diagnosis (http://www.seer.cancer.gov). Although the most common indication for liver resection in developed countries is metastatic CRC, surgery can only be performed in 20% patients, with the 5-year survival rate of 25%-40% despite adjuvant chemotherapy[4]. Regardless of this depressing scenario, a better understanding of tumor biology, combined with advances in molecular and cell biology, have opened up novel avenues of treating advanced CRC using immunotherapeutic strategies.

Tumor escape: Perverted local and systemic immune regulation by tumors

The cellular immune system has been endowed with powerful and at the same time toxic mechanisms designed to induce inflammation and cell destruction, which should be kept under tight control and guided precisely to the target tissues. Cytotoxic mechanisms are designed to recognize and destroy cells that are infected with viruses or other intracellular pathogens, whereas inflammation

is a vascular and leukocyte mediated local response that selectively directs the cellular and macromolecular elements of the innate and adaptive immune systems to the infected site. If properly aimed and enhanced, both immune functions can be therapeutically exploited to control and even eradicate malignant lesions[5]. Genetic and epigenetic changes involved in carcinogenesis generate antigens that are recognized by T lymphocytes in analogous fashion to microbial antigens[6]. Unfortunately, tumor cells in spite of being antigenic are very poorly immunogenic by themselves. Therefore, advanced cancer disease can impede any effort to induce antitumor immunity.

Genetically unstable cells can undergo genetic or epigenetic changes in order to escape a tumoricidal immune response in a "survival of the fittest" type of selection. The escape mechanisms may result from loss of antigen or antigen presentation as well as from active biosynthesis of immunosuppressive molecules[7,8]. These factors include TGF-β, VEGF, IL-8 and IL-10 which are known to cause significant inhibition of both innate and adaptive mechanisms of tumor immunity. Recent evidence points to activation of the transcription factor *Stat3* as a master switch in the control of various immunoevasive substances in tumor cells[9]. Moreover, intrinsic *Stat3* signaling in hemopoietic cells hindered their performance in tumor immunity including dysfunction of NK cells, granulocytes, and conventional DCs which become tolerogenic. Infiltration of tumors by effector T cells seems largely an inefficient process that may be related to poor expression of chemokines and vascular adhesion molecules in the malignant lesions[10]. Besides, the myeloid and lymphoid cells present in tumor stroma appear to be related more to the mechanisms of inhibition than to the activation of tumor immunity.

Indoleamine 2, 3 dioxygenase (IDO) catalyses the degradation of the essential amino acid tryptophan and synthesizes immunosuppressive metabolites[11]. Local up-regulation of the expression and activity of IDO in tumors and the draining lymph nodes can suppress T cell activation and is thought to facilitate the escape of tumor cells from the immune system[12]. Indeed, this enzyme depletes tryptophan and produces kynurenines locally in such a way that both mechanisms impair the function of T cells[13]. IFNs are the key factors upregulating IDO, thus generating a clever mechanism that becomes operational when tumors sense an active immune response in their neighborhood. There is recent evidence indicating that upregulation of IDO by colorectal cancer cells provides an immunosuppressive microenvironment created by tumors to promote cancer growth and spread[14]. We have observed in *in vitro* studies that the addition of IFN-γ to CT26 murine colorectal carcinoma cells induces IDO mRNA expression as well as IDO enzymatic activity, detected as kynurenine production (Figure 1).

Co-signaling molecules are cell-surface glycoproteins that can direct, modulate and fine tune T-cell receptor (TCR) signals[15]. The functional outcome of T cell activity upon its binding to a ligand on an adjacent cell membrane classifies co-signaling molecules as co-stimulators and co-inhibitors. Tumors can express co-inhibitory B7 family

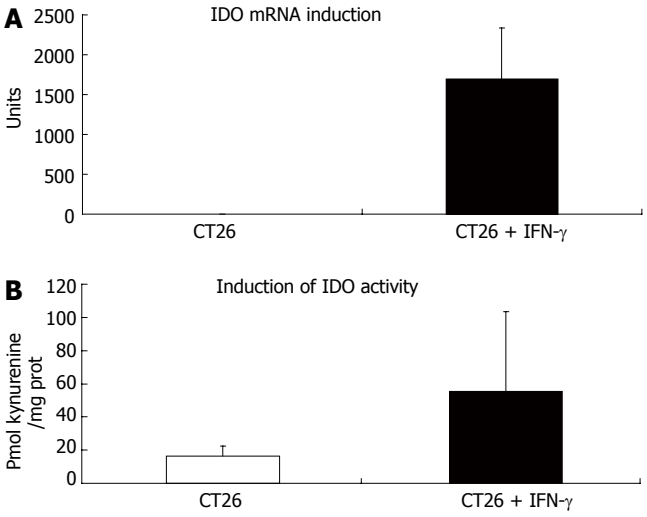

Figure 1 IFN-γ induces IDO mRNA and enzymatic activity in colon cancer cells. **A**: IDO mRNA was induced after 48 h stimulation with 1000 IU/mL of IFN-γ, as assessed by real time-PCR; **B**: In the same culture conditions, IDO activity was measured in CT26 cellular extracts as previously described by Takikawa *et al*[83].

members, such as B7-H1, B7-H4, and B7-1 (CD80) at a low density, which downregulates T cell activation and/or cytolytic activity[16,17]. Tumors can also induce B7-H1 and B7-H4 expression on tumor-associated macrophages (TAM)[18]. Myeloid suppressor cells can further inhibit anti tumor T cells *via* the production of nitric oxide by the enzyme arginase[19].

Regulatory T cells (T-reg) are important inhibitors of anti tumor immunity[20]. T-reg, characterized by the FoxP3 transcription factor, up-regulate a number of cell membrane molecules, including LAG-3, CTLA-4, GITR, and neuropilin. T-reg can inhibit effector T cell activation and function *via* T-T inhibition or inhibition of antigen presenting cells. There is experimental evidence to support a grim scenario in which T cells in tumor tissue or draining lymph nodes can be perverted into regulatory T cells[21]. Local production of TGF-β may be a key factor in transforming effector T cells locally into suppressive T-reg. Convincing data concerning the role of $CD4^+$ $CD25^+$ regulatory T cells in human cancer comes from the work of Curiel *et al*[22], who showed that the presence of such T-reg in advanced ovarian cancer correlated with reduced survival. Considering the role of T-regs as inhibitors of anti tumor immunity, it has been observed in murine models and in patients that prior host immunosuppression with chemotherapeutic agents (such as cyclophosphamide) can increase the efficacy of adoptive cell therapy as well as other kinds of immunotherapy[23]. The reason for this immunomodulatory effects is based, at least partially, on the elimination of $CD4^+$ $CD25^+$ T cells and the engraftment of specific cytotoxic T lymphocytes[24].

Experimental evidence with TCR transgenic mice clearly shows that tumor-reactive T cells can be tolerized to the point where there is no response to the surrogate tumor antigen. Tolerance results from presentation in the context of a DC that is not expressing high levels of costimulatory molecules and does not secrete cytokines such as IL-12, IL-15 and IFNs.

Chronic exposure to high levels of antigen drives T lymphocytes to a state of non-responsiveness termed "exhaustion". This phenomenon may play a role in impaired CD8 T-cell activity in response to persistent tumor antigens. In a way, the phenomenon of CD8 T-cell exhaustion is actually encouraging from the perspective of immunotherapy, since tumor-specific CD8 T-cells may be present and partially primed in a tumor-bearing host. The B7H1 and PD1 ligand receptor pair is a clear candidate to mediate and sustain exhaustion and offers an opportunity for therapeutic intervention.

In many cases however, a responsive TCR repertoire and tumor antigens coexist without signs of immunization or tolerization. Such a situation is termed immunological ignorance or indifference[25]. Ignorance can conceivably take place in two different ways. First, the quantity of antigen presented to the lymphoid tissue may be too small to induce immunity or tolerance. That would be ignorance/indifference at the priming phase of the immune response[26]. Second, studies in mice show that an expanded effector cell population respects tissues that are not inflamed[27,28]. This can be termed ignorance at the peripheral level that can occur in peripheral solid tumors[28,29].

The possibility of overcoming immunoescape

Immunotherapy, which is an intervention designed to increase anti-cancer immunity, remains an experimental discipline[30]. However several approaches including inducing and redirecting immunity to either the malignant cells or to critical components of the tumor stroma, such as the vasculature or the connective tissue, have been shown to profoundly impact disease progression in mouse models of cancer[31,32].

Therapeutic vaccination has been attempted in several ways. The immunogenic source can be autologous or allogenic malignant cells that are modified to increase their immunogenicity[33]. *Ex-vivo* or *in vivo* gene transfer of cytokines and other immune-potentiating molecules is a promising strategy. Alternatively, many experimental protocols rely on *in vitro* culture/differentiation of DCs manipulated in such a way that they artificially present tumor antigens[34]. However, the promising results in mouse models have not been replicated in clinical trials. In spite of this drawback there is ample biological evidence in humans that there is an increase in the numbers and activity of lymphocytes against the vaccinating antigen, although such increases fail to reach by 1-2 logs the levels of T cell immunity observed in viral infections.

Adoptive T cell therapy with activated T lymphocytes reaches higher levels of circulating antitumor T cells[35]. These techniques are based on *ex-vivo* reactivation and expansion of cloned or polyclonal cultures of tumor reactive T cells. After culture, T cells are reinfused into the patient along with IL-2. Three important concepts have gained experimental support: (1) polyclonal cultures that recognize several antigen specificities improves the outcome, and the development of tumor-escape antigen loss variants are less likely to occur, (2) co-infusion of both CD4 and CD8 tumor reactive T cells improves antitumor activity, and (3) treatment with lymphodepleting chemotherapy before reinfusion increases the duration and *in vivo* re-expansion of the infused T cells. This is due to both depletion of regulatory T cells and decrease in the competition for T cell homeostatic survival factors such as IL-15 and IL-7. Adoptive T cell therapy probably will benefit much more from the availability of clinical grade IL-15, which can condition the infused cells and sustain their function on administration to the patient.

The sense that chemotherapy and immunotherapy are incompatible is a fading paradigm in tumor immunotherapy. It used to be reasoned that if T cell responses require cell expansion, active or adoptive immunotherapy could not be used in combination with chemotherapy drugs that are selectively toxic for dividing cells. Several lines of experimental evidence suggest otherwise. In fact, there are a number of mechanisms that define additive and synergistic effects: (1) tumor cell destruction makes tumor antigens available for cross presentation by DCs, (2) there is decrease in regulatory T cells, and (3) there is reduced competition for T-cell homeostatic growth factors during/after active immunization. Local destruction of tumors followed by injection of proinflammatory substances holds much promise according to preclinical data and probably represents the simplest method of converting tumors into tumor vaccine.

Immunostimulatory monoclonal antibodies for the treatment of colorectal carcinoma

Immunostimulatory mAbs directed to immune receptors have emerged as a new and promising strategy to fight cancer[36]. In general, mAbs can be designed to bind molecules on the surface of lymphocytes or antigen presenting cells to provide activating signals (e.g., CD28, CD137, CD40 and OX40)[36]. On the other hand, mAbs can also be used to block the action of surface receptors that normally downregulate immune responses (CTLA-4 and PD-1/B7-H1). In combined regimes of immunotherapy, these mAbs are expected to improve therapeutic immunizations against tumors as observed in preclinical studies.

Anti-4-1BB (agonistic anti-CD137) is one of the most interesting mAbs tested as anti-cancer molecules in preclinical studies[36]. 4-1BB is a member of the tumor necrosis factor/nerve growth factor family of receptors and has a natural ligand (4-1BBL) that is expressed on activated T lymphocytes as well as on NK cells and dendritic cells[37]. This mAb, which acts against CD137, has the ability to stimulate potent antitumor responses[38] and, paradoxically, ameliorates autoimmune manifestations in mice[36]. On the other hand, therapy with mAbs against CTLA-4, which block the inhibitory action of CTLA-4 on T-cells, is capable of inducing antitumor responses in mice as well as in humans but is accompanied with adverse events in the form of autoimmune reactions[39].

Kocak *et al*[40] took advantage of both the mAbs and showed that the combination of CTLA-4 and 4-1BB acts synergistically in the eradication of MC38 colorectal carcinoma after stimulation of a potent antitumor immune response. It was observed that this antitumoral effect

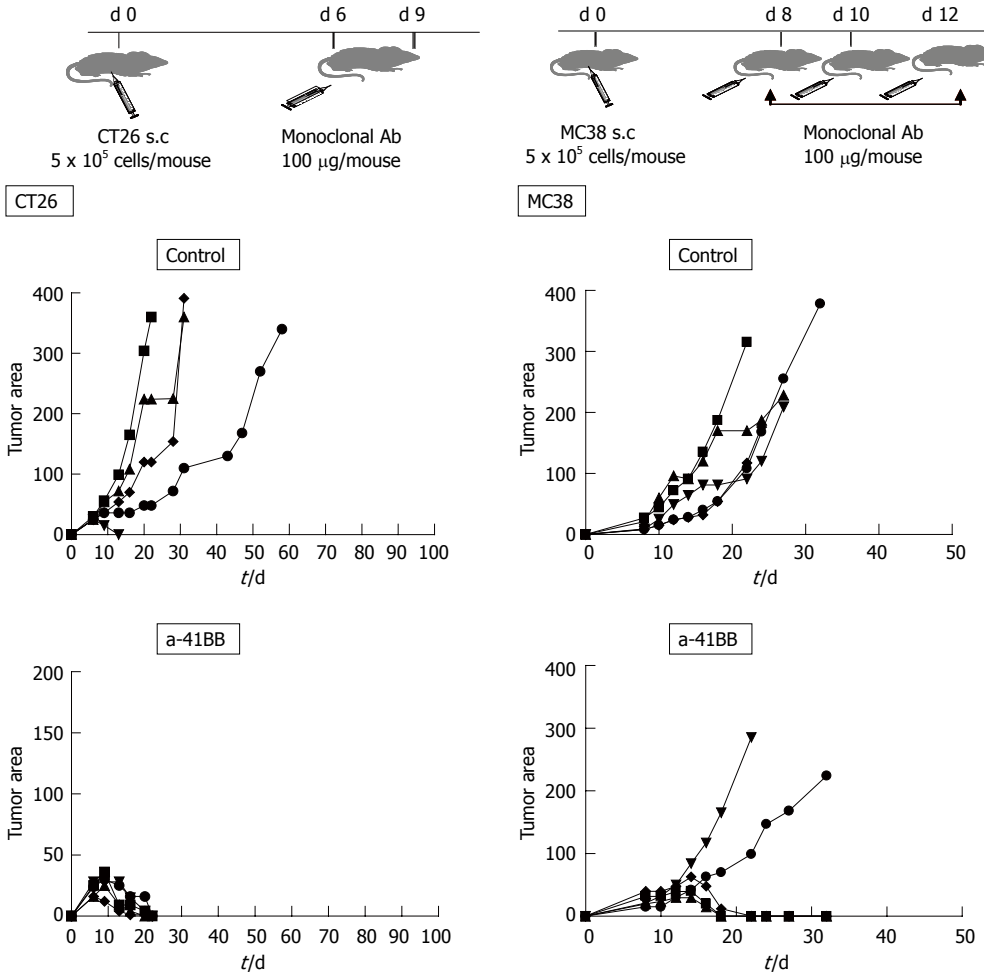

Figure 2 Systemic treatment with agonist anti-CD137 monoclonal antibodies eradicates transplanted murine colon cancers. Mice subcutaneously grafted with 5 x 10^5 CT26 or MC38 cells were treated with anti-CD137 (2A) mAb or polyclonal rat IgG as a control. Sequential follow up of tumor size (mean diameter) is depicted for individual mice.

is critically dependant on the presence of $CD8^+$ T-cells induced after treatment[40]. However, we did not observe such a synergy in the same experimental model (A Arina *et al*, unpublished observations).

In our studies in mice, we used the MC38- and CT26-derived tumor model (colorectal carcinoma cell lines) to explore the antitumor effect of repeated systemic injections of agonistic anti-CD137 (anti-4-1BB) mAbs. As a result of the amplification properties of anti-CD137 antibodies on CTL immune response, this treatment was able to induce tumor eradication in 3 out of 5 mice bearing CT-26 tumors and in 3 out of 5 animals with MC38 nodules (Figure 2).

CD137 stimulation can be achieved not only by direct administration of mAbs in monotherapy, but also in the context of different combinations usually including immunostimulatory cytokines. For example, simultaneous gene transfer of local-membrane bound 4-1BB ligand and IL-12 results in successful eradication of advanced colorectal liver metastasis induced in mice[41]. In a similar line of work, Martinet *et al*[41] demonstrated that the combination of 4-1BB costimulation using an adenovirus expressing membrane-bound 4-1BB-L with another adenovirus expressing IL-12 genes induced a potent antitumor response in mice with colorectal carcinoma. Systemic administration of soluble Ig-4-1BB ligand gave rise to a stronger T-cell immune response compared to local gene transfer[42]. It appears that anti-4-1BB can upregulate a formerly weak immune response, but it fails to initiate an immune response if it was nonexistent initially[43].

Systemic treatment with anti CTLA-4 mAb increased the number of CTLs and caused complete tumor regression in established colorectal carcinoma in mice[44]. Another attractive immunostimulatory combination was recently examined by Tirapu *et al*. These workers searched for strategies to enhance the efficacy previously achieved by intratumoral injection of DCs engineered to secrete IL-12 in a mouse model of colorectal carcinoma (using MC38 cell line). They were able to induce a systemic immune response (measured by IFN-γ ELISPOT assay) that eradicated large and metastatic tumor lesions using a combination of systemic anti-CD137 mAb and IL-12 producing semiallogeneic DCs injected intratumorally[45]. This study offers a promising technique of enhancing the efficacy of DC-based strategies currently been tested in clinical studies[46].

GENE TRANSFER OF IMMUNOSTIMULATORY MOLECULES AND GENETIC VACCINATION

Several cytokines (e.g., IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IFN-γ, TNF-α and GM-CSF) demonstrate an ability to increase anti-tumor immunity when expressed by cancer

Table 1 Gene transfer of immunostimulatory molecules and genetic vaccination

Cytokine	Vector	Clinical application	Mechanism	Ref.
IL-2 + IL-12	Ad	No	CTLs	10
IL-10	Retrovirus	No	$CD8^+$	54
TNF-alpha	Ad	Yes	Antiangiogenic, bystander effect	75
HLA-B7/b2 microglobulin	DNA	Yes	CTLs	76
IL-12	Ad	Yes	NK, $CD4^+$, $CD8^+$	46
IL-12 + IL-10	Retrovirus	No	$CD8^+$, $CD4^+$, NK, Macrophages, Neutrophils	55
IL-2	Ad, retrovirus	Yes	CTLs	59
CCL21/LIGTH	Ad	No	DC, $CD8^+$, Macrophages	61

Ad: Adenovirus; DNA: Plasmid DNA; CTLs: Cytotoxic T lymphocytes; NK: Natural killers.

cells[47]. However, systemic administration of recombinant cytokines has limitations because of their short half-life, production difficulty and toxicity. Gene therapy appears to be a novel strategy that may help in delivering therapeutic genes locally, as well as the possibility of controlling transgene expression using specific and regulatable promoters[48] (Table 1).

Currently, we consider two principal approaches to the transfer of immunostimulating molecules inside tumors in order to facilitate immunity against colorectal cancer[47]: (1) *in vivo* injection of vectors expressing cytokines/costimulatory molecule genes into the tumor milieu (may be the most straightforward technique), and (2) tumor cells, DC and lymphocytes can be transduced *ex vivo* with vectors encoding cytokines/costimulatory molecules and re-administered into the host. One of the aims of these strategies is to induce high tumoral or peritumoral production of transferred cytokines, to promote localized regional inflammation (to stimulate innate anti-tumor response), and to induce systemic immunity capable of eliminating disseminated disease.

One of the most extensively studied cytokines in cancer treatment is interleukin-12 (IL-12), which has been shown to have significant antitumor activity against a wide panel of experimental malignancies. IL-12 promotes antitumor immunity because of its ability to activate cytotoxic T lymphocytes (CTLs), natural killer (NK cells) and Th1 response[49,50]. Moreover, IL-12 has antiangiogenic effect, dependent on Interferon gamma (IFN-γ) Inducible Protein 10 (IP10) that facilitates its anticancer effect through different mechanisms[51,52]. It is well known that systemic therapy with rIL-12 protein carries the risk of severe toxicity because of the stimulation of large quantities of IFN-γ, with the potential for individually heterogeneous susceptibility[53].

It has been observed that a combination of immunostimulatory genes may achieve superior therapeutic effects. Narvaiza *et al*[51] demonstrated that intratumoral administration of an adenovirus encoding IL-12 (AdIL-12) together with another adenovirus encoding the chemokine IP-10 (AdIP-10) results in marked antitumoral synergy leading to eradication of metastatic colorectal carcinomas. In this study, the authors used vectors in doses that were not effective when given separately. Moreover, this strategy allowed reduction in the dose of AdIL-12 without losing its anti-tumor efficacy and with less risk of IL-12-related toxicity[51]. The underlying principle of combining AdIL-12 and AdIP-10 is based on the prospect of attracting lymphocytes to tumors expressing IP-10 and to activate them by simultaneous infection of the tumor with AdIL-12.

It is well known that IL-12 has the ability to induce a Th1 type of immune response. By contrast, IL-10 is mainly expressed by Th2 cells and downregulates the production of IL-12 by antigen presenting cells, thus decreasing Th1 activity[51]. However, it has been observed that IL-10 enhances IL-2-induced proliferation and differentiation of $CD8^+$ T-cells[29]. Adris *et al*[54] showed that inoculation of mice with tumor cells expressing IL-10 inhibits the establishment of colorectal carcinoma cells and induces a T cell-mediated tumor suppression in the context of a systemic Th2 response. In an effort to treat colorectal carcinomas using both cytokines, Lopez *et al*[55] have shown that tumor cell vaccines producing both IL-10 and IL-12 act synergistically to eradicate established colorectal cancer (CT26 cell line) and, surprisingly, mammary carcinomas as well. The authors also observed that the antitumor effect of the combined immunotherapy was mainly dependent on $CD8^+$ cells.

In addition to IL-12, heat shock proteins (HSPs) also have the ability to stimulate antigen-presenting cells and induce a Th1-type response. HSP have been employed as an adjuvant to facilitate the induction of specific immunity. Moreover, HSPs have been evaluated in clinical studies as an adjuvant in combination with BCG (Bacille Calmette-Guerin) and HPV16E7 in patients with papillomavirus-related carcinoma[56]. Wu *et al*[57] demonstrated that vaccination of transgenic mice with HSP70-like protein (Hsp70L1) fused with a fragment of carcinoembryonic antigen (CEA576-669) induced the maturation of DCs, with a strong specific CD8 T cell response and *in vivo* antitumor activity in mice.

Systemic administration of recombinant IL-2 has been used in clinical practice in patients with metastatic renal carcinoma and malignant melanoma, although with low efficacy and high toxicity[58]. Among other functions, IL-2 is necessary for the survival of activated T cells and is employed in large doses in protocols were immune cells are adoptively transferred to cancer patients. Adenovirus containing mouse IL-2 cDNA can be injected into tumors, and in combination with a suicide gene (herpes simplex virus thymidine kinase vector) can be a powerful tool in the treatment of metastatic colon carcinoma of the liver[59].

One of the synergistic combinations include a chemokine plus a T-cell-activating cytokine designed to promote the attraction and activation of infiltrating immune cells (attraction theory). Macrophage inflammatory protein 3 (MIP-3) is a chemokine mainly secreted by activated macrophages, which attracts leukocytes to inflammatory foci with selectivity for tisular

DCs. The combination of two adenoviruses, one encoding MIP-3 (Ad MIP-3) and the other IL-12 genes (AdIL-12) given intratumorally in mice with colorectal carcinoma eradicates nearly 90% of subcutaneously implanted tumors[60]. Similarly, co-expression of the chemokine CCL21/secondary lymphoid tissue chemokine and a costimulatory molecule LIGHT in colon carcinoma cells (CT26) resulted in significantly reduced tumor growth in mice. A markedly increased infiltration of mature DCs and $CD8^+$ T cells was observed in the tumor mass, and the splenocytes showed a potent CTL activity against CT26 tumor and IFN-γ production. These results suggest that combined treatment with CCL21 and LIGHT is capable of inducing a synergistic antitumor effect[61].

Dendritic cell-based immunotherapy

Dendritic cells (DCs) are leukocyte populations that present antigens captured in peripheral tissues to T cells *via* both MHC class Ⅱ and I antigen presentation pathways[62]. DC maturation is referred to as the status of DC activation at which such antigen-presenting DCs leads to T-cell priming, while its presentation by immature DCs results in tolerance[63]. DC maturation is chiefly caused by biomolecules with microbial features detected by innate receptors (bacterial DNA, viral RNA, endotoxin, *etc*), pro-inflammatory cytokines (TNF, IL-1, IFNs), ligation of CD40 on the DC surface by CD40L, and substances released from cells undergoing stressful cell death.

It is well known that DCs are potent inducers of immune responses and the activation of these cells is a critical step for the induction of antitumoral immunity. We successfully tested a technique designed to take advantage of the therapeutic effect of IL-12 infecting DCs *ex vivo* with an adenovirus that expresses IL-12 genes (AdIL-12), and injecting the engineered cells into colorectal carcinomas in mice[64]. This strategy has proved to be exceptionally effective in eliminating neoplastic nodules and in eliciting anti-tumor immunity. This strategy is also effective in mouse models when DCs are transfected to express IL-7[65] and IL-15[66].

Transfection of DCs with mRNA is a promising antigen-loading technique of stimulating strong antitumor immunity. Chu *et al*[67] transfected RNA from CT26 colorectal adenocarcinoma to the bone marrow-derived monocytes and obtained strong specific CTL activity *in vivo*. Saha *et al* [68] showed that immunization of CEA transgenic mice with bone marrow-derived mature dendritic cells loaded with the antidote antibody 3H1 (which mimics CEA) resulted in a CEA-specific immune response and suppression of colon carcinoma cells (expressing CEA) in nearly 100% of mice, whereas only 40% of experimental mice immunized with dendritic cells loaded with CEA were protected from tumor growth.

Furumoto *et al*[69] injected MIP-3 chemokine together with CpGs into colorectal carcinomas in order to activate *in vivo* dendritic cells without *ex vivo* manipulation. These workers observed an increase in the number of activated DCs in tumors that were eradicated through specific T cell-mediated antitumor response.

CD40L, a costimulatory molecule expressed on activated $CD4^+$ T cells, acts on B cells and DCs, and plays a key role both for maturation of antibody responses and for CTL induction. Investigators from Crystal's group demonstrated in studies on mice, synergy in the eradication of subcutaneously implanted CT26 when treated with a combination of intratumor injection of an adenovirus expressing CD40-L with DCs or when each treatment was applied sequentially[70,71].

Morse *et al* reported a phase I clinical trial in which autologous dendritic cells loaded with carcinoembrionic antigen RNA (peptide CAP-1) were administered to patients with resected liver metastases from colorectal carcinoma. The procedure was well tolerated, and one patient had a minor response, and one showed stable disease[72]. With the aim to expand the presence of circulating DCs (DC mobilization), Fong *et al*[73] in a phase I study used the hematopoietic growth factor Flt3 ligand prior to the injection of CEA-derived peptide loaded DCs in 12 patients with colon or non-small cell lung cancer. One patient had a mixed response while two showed stable disease.

DCs engineered to produce IL-12 have been shown to induce potent anti-tumor responses. We have recently completed a phase I clinical trial which involved intratumor injection of monocyte-derived autologous dendritic cells transfected *in vitro* with an adenovirus encoding human IL-12 in patients with metastatic gastrointestinal carcinomas[46]. The main objectives of the trial were to assess feasibility and safety, and secondarily to determine biologic and clinical responses. We observed that this strategy was safe and well tolerated, with injection of up to 50×10^6 dendritic cells. Five patients showed increased NK activity and 4 showed augmented intratumor $CD8^+$ T-cell infiltrate. One partial response and two stabilizations were observed. The reasons for the weak antitumor response were explored. It appears that DCs can be retained inside malignant tissue by means of high intratumor concentrations of IL-8. Besides, scintigraphic tracking of intratumorally injected DCs labelled with ^{111}In indicated the retention of DCs inside malignant lesions in patients with digestive carcinomas[74].

CYTOKINE GENE TRANSFER FOR COLORECTAL CARCINOMA IN CLINICAL SETTING

Over 1100 gene therapy clinical trials have been carried out around the world and almost 70% of them were directed at the treatment of advanced or metastatic cancer. In clinical trials, cytokine and tumor antigen genes represent 42% of the genetic material that is transferred (for details see: www.wiley.co.uk/genmed/clinical). In the following section, we focus on some of the most important cytokines currently under clinical investigation in immunogene therapy of colorectal carcinoma.

The encouraging results obtained with the administration of non-replicative adenovirus encoding for IL-12 genes in several experimental models of gastrointestinal cancers (for review see reference[1])

prompted us to initiate a clinical trial at the University of Navarra in patients with advanced gastrointestinal carcinomas[46]. Patients with hepatic tumors (either primary or secondary colorectal carcinomas) were treated intratumorally in a dose-scale fashion with an adenovirus encoding human IL-12 genes. This strategy was safe and well tolerated with only minor side effects. Biological activity was observed in some patients (e.g., rise in serum levels of IFN-γ, infiltration of tumors by $CD8^+$ T cells and induction of neutralizing anti-adenovirus antibodies). Partial tumor regression was observed in one patient and stable disease in 30% patients. Reduction in the gap between doses in the same patient, or application of the vector as neoadjuvant therapy before tumor resection are some of the potential approaches to increase the efficacy of this treatment strategy.

The dose-limiting toxicity of large systemic concentrations of TNF- has led to a decline in its use in cancer patients. By contrast, local gene transfer of this cytokine using an adenovirus (TNFerade®) may reduce the systemic effects. TNF- gene under the control of an early growth response 1 (EGR-1) promoter followed by external beam radiation allows the control of TNF- release. Promising antitumor activity without any significant toxicity was observed in patients with solid tumors[75]. TNFerade® in combination with capecitabine and radiation therapy is now being tested in a phase Ⅱ clinical trial on patients with rectal cancer, before surgical resection.

Rubin *et al*[76] showed that direct gene transfer of HLA-B7 and 2-microglobulin, which together form a MHC-I complex, into the liver of patients with metastatic colorectal carcinoma is a feasible and safe procedure. These workers used a single plasmid construct that encodes for both genes in a formulation containing the lipid complex DMRIE-DOPE (Allovectin-7®). Genes transfected into tumors were detected by PCR in 14 out of 15 patients, however. the clinical results have not published. It should be noted that better results have been obtained in patient with melanoma.

With the advent of agents such as irinotecan and oxaliplatin, chemotherapy has made some progress in the treatment of colorectal carcinoma. The use of biological therapy with monoclonal antibodies against VEGF and EGFR has been shown to benefit a small proportion of patients[77]. Immunotherapy in different forms should be tested in addition to the conventional treatment regimens which improve patient survival.

Concluding remarks and future directions

There is a striking correlation between lymphocyte infiltration in colorectal cancer and the overall outcome of the disease[78,79]. Indeed, the density of T cells close to the tumor cells in the primary tumor is a better predictor of survival in these patients than traditional staging based on tumor size and spread[80]. According to this study, patients whose tumors contained large numbers of CD3-positive T cells, had a 5-year survival rate of 73%, compared with 30% in patients with low density of these cells.

There are important conclusions to be drawn from this study: (1) There is much natural immune pressure on colon cancer that may control the disease successfully in many patients, (2) The immune pressure possibly selects tumor variants that eventually escape immune control, (3) Artificial augmentation of the immune response may tilt the balance towards a curative response at least in some cases.

Immunotherapy intervention requires tumor-debulking and therefore should be combined with surgery and chemotherapy. To make the most of immunotherapy, this technique should be tested on patients whose tumors have been completely resected but are at high risk of relapse. For instance, our current efforts are focused on patients whose liver metastases have been resected surgically and are receiving adjuvant chemotherapy. In these patients, measures to induce/enhance cellular antitumor immune responses may confer a clinically significant delay in tumor relapse. Moreover, the complete removal of any detectable disease greatly diminishes the immunosuppressive mechanisms that may otherwise be induced by the cancer, while the surgical samples provide a rich antigenic source for immunization. Interference with the immunosuppressive mechanisms is clinically feasible with the use of low doses of cyclophosphamide[81] and other such mechanisms may become clinically available in the near future.

In our opinion, it is at the stage of minimal residual disease when immunotherapy should be fully deployed with a combination of strategies comprising of immunization with different tumor antigens and amplification techniques using cytokines or/and immunostimulatory monoclonal antibodies[82].

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Is there a genetic signature for liver metastasis in colorectal cancer?

Cristina Nadal, Joan Maurel, Pere Gascon

Cristina Nadal, Joan Maurel, Pere Gascon, Servei Oncologia Mèdica, Institut Clínic de Malalties Hemato-Oncològiques, Hospital Clínic de Barcelona, c/Villarroel, 170, Barcelona 08036, Spain
Correspondence to: Cristina Nadal, Servei Oncologia Mèdica, Institut Clínic de Malalties Hemato-Oncològiques, Hospital Clínic de Barcelona, c/Villarroel, 170, Barcelona 08036, Spain. cnadal@clinic.ub.es
Telephone: +34-93-2275402 Fax: +34-93-4546520
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Abstract

Even though liver metastasis accounts for the vast majority of cancer deaths in patients with colorectal cancer (CRC), fundamental questions about the molecular and cellular mechanisms of liver metastasis still remain unanswered. Determination of gene expression profiles by microarray technology has improved our knowledge of CRC molecular pathways. However, defined gene signatures are highly variable among studies. Expression profiles and molecular markers have been specifically linked to liver metastases mechanistic paths in CRC. However, to date, none of the identified signatures or molecular markers has been successfully validated as a diagnostic or prognostic tool applicable to routine clinical practice. To obtain a genetic signature for liver metastasis in CRC, measures to improve reproducibility, to increase consistency, and to validate results need to be implemented. Alternatives to expression profiling with microarray technology are continuing to be used. In the recent past, many genes codifying for proteins that are directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have been linked to mechanisms of liver metastases in CRC.

Key words: Colorectal cancer; Liver metastasis; Genetic signature; Expression profile; Arrays

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer, with a worldwide incidence of almost a million cases annually in both males and females[1]. Despite advances in screening, approximately 25% of patients have initially detectable liver metastases (synchronous metastases), and an additional 25% of patients will develop liver metastasis during the course of their disease (metachronous disease)[2]. Of all patients who die of advanced colorectal cancer (ACRC), 60% to 70% show liver metastasis[3]. Metastatic spread to the liver is the major contributor to mortality in patients with CRC.

CRC is a genetically heterogeneous and complex disease. Initially, two major pathways were described as being responsible for the CRC tumorigenic process: the chromosomal instability pathway and the microsatellite instability pathway. The chromosomal instability or classical pathway accounted for 85% of the tumorigenic processes and was mainly characterized by the sequential allelic losses on chromosomes 5q (APC gene), 17p (TP53) and 18q (DCC/Smad4). The microsatellite instability pathway (MNI), which is associated with the mutator phenotype, only accounted for 15% of the carcinogenic processes. Recently, it has been shown that colorectal carcinogenesis is much more complex, involving new pathways, such as the serrated, the TGFβ/Smad and epigenetic pathways, and also non-pure or mixed pathways[4-6].

The general mechanisms of tumorogenesis also include metastasis generation mechanisms. But, is the knowledge of CRC tumorigenic pathways extensible to metastasis generation? What do we really know about the molecular determinants of liver metastases formation in CRC?

MECHANISMS OF LIVER METASTASIS

Colorectal liver metastasis, or dissemination and colonization by colorectal tumor cells coming from the primary CRC to the liver, is a complex process and has many different steps. In order to metastasize, tumor cells detach from the primary tumor, invade and migrate through the stroma and intravasate into the lymphatic and/or venous vessels. With either as the vasculature entrance, cells will mainly end up travelling through the portal vein system. During transportation they manage to survive mechani-

Table 1 Summary of gene expression profile studies related to CRC liver metastasis

Source for transcription profile comparisons	Authors	Signature	Prediction
Primary tumors (Stage Ⅱ and Ⅲ)	Bertucci *et al*[11]	46 gene set	Lymph Node (+)
Primary tumors (Dukes C)	Arango *et al*[12]	Two different gene sets	Survival
Primary tumors (Stage Ⅱ and Ⅲ)	Barrier *et al*[13]	30 gene set	Lymph Node (+)
Primary tumors (Stage Ⅱ and Ⅲ)	Komuro *et al*[14]	Gene set	Stage Classification
Primary tumors (Stage Ⅱ and Ⅲ)	Kwon *et al*[15]	60 gene set	Lymph Node (+)
Primary tumors (Dukes B)	Wang *et al*[16]	23 gene set	Recurrence
Primary tumors (Stage Ⅱ to Ⅳ)	D´Arrico *et al*[17]	37 gene set	Distant Recurrence
Primary tumors and matched metastases	D´Arrico *et al*[17]	GnT-IV gene[1]	Liver Metastasis
Primary tumors and matched metastases	Koehler *et al*[18]	Not found	Liver Metastasis
Primary tumors and matched metastases	Agrawal *et al*[20]	11 gene set	Metastasis (including liver)
CRC cell lines[2]	Hegde *et al*[21]	11 gene set	Metastatic potential
CRC cell lines[2]	[11-14,16,17,22]	Individual genes[3]	Metastatic potential

[1]Mannosyl (alpha-1, 3-)-glycoprotein beta-1, 4-N-acetyl-glucosaminyl-transferase, which was found up-regulated in CRC liver metastases compared to primary CRC tumors; [2]Comparing SW480 to SW620; [3]Down-regulation of Cadherin 17 (CDH17)[11,22], Insulin-like growth factor 2 (IGF2)[14,17], Tyrosine 3-monooxygenase/tryptophan-5-monooxygenase activation protein (YWHAH)[12,16], DEK oncogene (DEK)[11,12] and GATA binding protein (GATA6)[11,14], up-regulation of Linker for activation of T cells (LAT)[14,16] and Protein Kinase, cAMP dependent, catalytic alpha (PRKACA)[12,14], and altered expression of IQ motif containing GTPase activating protein 1 (IQGAP1)[11,12], Tumor protein 53 (TP53)[11,12], Oligoadenylate synthetase 1 (OAS1)[11,12], Interferon regulatory factor (IRF2)[11,14], Retinoic acid receptor beta (RARB)[11,12] and Programmed cell death 10 (PDCD10)[12,13].

cal stresses and escape from the immune system. Some stresses keep acting once cells arrest in the liver capillaries. Some of the arrested cells manage to adhere to endothelial cells, contact the extracellular matrix and extravasate to the surrounding tissues. Kupffer cells, belonging to the monocyte-macrophage system, are a perfect barrier to unwanted hosts. Being in the liver parenchyma, tumor cells establish crosstalk with the stroma and create a microenvironment. Only if this microenvironment is favourable to tumor cells, signals of proliferation and neoangiogenesis will lead to macroscopic liver metastasis formation[7-9]. Even though liver metastasis accounts for the vast majority of all cancer deaths in patients with colorectal cancer, fundamental questions about the molecular and cellular mechanisms of liver metastasis still remain unanswered.

Genetic signatures: The breakthrough

The availability of DNA array technology, allowing genome-wide analyses of gene expression, has been providing new insights on the determination of gene expression or transcriptional profiles. Expression profiling studies in CRC have mainly focused on comparisons of normal mucosa, adenoma and primary carcinomas. Few studies have thrown light on differences between primary tumors and metastases. For this reason, in contrast to the many molecular alterations involved in the CRC adenoma to carcinoma step characterized to date, comparatively little information is available on the possible mechanisms of metastases, with even less for liver specific metastases[10].

There are two different aspects of metastasis to consider: metastatic ability and tropism or organ-specificity. Metastatic ability accounts for the potential to establish a distant secondary tumor. Organ-specificity or tropism means the capacity of this happening in a specific type of tissue. The ability to metastasize together with the specificity for it to happen in one organ and not in another can be genetically marked by what is called a metastatic signature. Studies looking at mRNA or protein levels take into account expression regulation, splicing mechanisms, epigenetic phenomena, and the complexity of post-translational changes or modifications. A metastatic signature, therefore, is not a gene list but is a translation of the functional status of gene expression. Metastatic signatures are gene expression patterns conditioned by both an intrinsic gene composition and phenomena regulating expression.

In order to determine metastatic signatures by microarray technology in CRC, three different strategies have been followed (Table 1). The first approach consists of comparing transcriptional profiles of primary CRC from metastasis-free patients to those affected by metastatic spread during a 5-year follow-up period. The main goal is finding gene expression profiles as prognostic markers of metastatic spread. Identification of a gene set capable of classifying CRC patients according to prognosis or 5-year survival rate was carried out by Bertucci *et al*[11]. A total of 219 genes and 25 genes were found to be respectively down- and up-regulated in metastatic samples when compared to non-metastatic patients. Moreover, a 46 gene set signature was isolated, discriminating between CRC with and without lymph node metastases. Arango *et al*[12] checked the expression profile of Dukes C CRC and reported two different signatures according to survival. Barrier *et al*[13] built an accurate 30-gene tumor-based prognosis predictor for stage Ⅱ and Ⅲ colon cancer patients, based on gene expression measures. The group of Komuro *et al*[14] analyzed gene expression profiles in a total of 89 CRC. After stratifying according to right and left locations, they were able to extract gene expression profiles characteristic of the presence versus absence of lymph node metastasis with an accuracy of more than 90%. Kwon *et al*[15] analyzed the gene-expression profiles of colorectal cancer cells from 12 tumors. Sixty genes possibly associated with lymph node metastasis in CRC were selected on the basis of clinicopathological data. Wang *et al*[16] analyzed RNA samples from 74 patients with Dukes' B CRC. Gene expression profiling identified a 23-gene signature that predicted recurrence. This signature was validated in 36 independent patients. The overall

performance accuracy was 78%. D´Arrico *et al*[17] compared the transcriptional profiles of 10 radically resected primary CRCs from patients who did not develop distant metastases within a 5-year follow-up period with those of 10 primary/metastatic tumor pairs from patients with synchronous liver metastases. The study was conducted on laser-microdissected bioptic tissues. Arrays of 7864 human cDNAs were utilized. Non-metastasizing primary tumors were clearly distinct from the primary/metastatic tumor pairs. Of 37 gene expression differences found between the 2 groups of primary tumors, 29 also distinguished nonmetastasizing tumors from metastases. The gene encoding for mannosyl (alpha-1, 3-)-glycoprotein beta-1, 4-N-acetyl-glucosaminyl-transferase (GnT-IV) became significantly upregulated in primary/metastatic tumor pairs ($P < 0.001$), supporting the existence of a specific transcriptional signature distinguishing primary CRCs with different metastatic potential[17].

The second approach consists of comparing gene expression in primary tumors with their matched metastases. Studies comparing gene expression between primary and corresponding metastases indicate that there is a high transcriptional resemblance. The above mentioned study found a striking transcriptional similarity between primary tumors and their distant metastases[17]. Another study by Koehler *et al*[18] determined expression profiles from 25 CRCs and 14 corresponding liver metastases using cDNA arrays containing 1176 cancer-related genes. Most primary tumors and matched liver metastases clustered together. A specific expression signature in matching metastases was not found, but a set of 23 classifier genes with statistically significant expression patterns in high- and low-stage tumors was identified. Gene expression studies in breast cancer also support the notion that primary tumors genetically resemble their matched metastases more than their primary counterparts[19]. Agrawal *et al*[20] found a signature of 11 markers for tumor progression when comparing gene expression among different stages, including liver metastases in a total of 60 samples.

Expression profiling using CRC cell lines with different metastatic potential is another approach[21,22]. Studies using cDNA microarrays have identified genes that are differentially expressed in primary *versus* metastatic CRC cell lines. Differential expression of 11 genes has been found in SW480 and SW620 CRC cell lines[21]. Unfortunately, metastatic signatures described in the above mentioned studies do not show much in common. Gene expression patterns do not overlap enough to show consistency. Only a few genes reported in at least two independent studies have been linked to metastatic ability (Table 1).

It is interesting that no expression profile has been specifically linked to liver metastases in CRC. Apart from gene expression profiling, other techniques, such as genomic profiling, have also been used to determine metastatic ability in CRC. Genomic analyses of primaries and their matched metastases[23] showed that CRC primary tumors resemble their corresponding metastases. Array-based comparative genomic hybridization (CGH) was used to detect genetic alterations in CRC that predicted survival after liver resection[24]. Genome wide copy number analysis revealed the involvement of Cycline D3 in liver metastases formation in CRC[25].

Genetic signatures: Handicaps and pitfalls

When determining metastatic expression profiles or signatures with array technology, several confounders have to be taken into account. Studies have employed important methodological differences, which are mainly due to the use of different array platforms (Affymetrix, cDNA nylon membranes) or experimental conditions. Tissue sampling is almost always an issue in this regard. Availability of frozen tissues is not the norm in many institutions. Formalin-fixed or paraffin-embedded tissues usually yield low quality RNA and/or DNA. The creation of frozen-tissue tumor banks is rapidly increasing. Also methodologies for RNA isolation can lead to different results. The number of samples used varies enormously in different studies. Relatively small cohorts of tumors have been analyzed in some studies, especially if they include the analysis of matched metastases. Selection of homogeneous samples among heterogeneous tumors can often be a problem. Anatomical localization (right *vs* left sided, colon *vs* rectum) and genetic instability status (MSI/classical) may justify the variability of CRC gene expression profiles characterized to date. Macrodissection techniques include tumor tissue with both tumor cells and tumor stroma and valid tissue samples should be at least 50% tumor cells. One of the major criticisms of "metastatic signature" -seeking studies is the fact that tumors are analyzed as a whole, mixing tumor cells with microenvironment and stroma components. Certainly, data coming from these experiments is a mixture representing gene expression of tumor cells, stroma cells as well as their interactions. Moreover, expression data can be highly conditioned by the host genetic background. Resulting data can be highly interesting in terms of defining prognosis, but not in understanding the mechanisms of metastasis generation. Microdissection techniques help to avoid this problem. Laser capture microdissection (LCM) allows isolation of only tumor cells and is considered the gold standard in microdissection procedures[26]. It is a time-consuming technique and it is not available at all institutions. Other strategies include subtracting non-tumor cell signatures from gene expression data[27]. It is still unclear whether the analysis of pure tumor cell populations will lead to an appropriate result in terms of prognostic value.

Description of metastatic signatures has been done on the basis of transcription analysis of tumors. Data from DNA microarray analysis is often overwhelming and mixed. Analysis of differentially expressed genes can be altered by the use of different criteria to define low-quality spots, different normalization procedures, different baseline references for ratio calculations, and arbitrary criteria for cut-off values applied to fold-change and significance level. Commonly, quantitative levels of expression are the basis for filtering the raw data. During filtering, information coming from qualitative data can be lost[10]. Moreover, the final data set has to be interpreted and integrated to make sense in biological terms. This step is highly subjective and probably often leads to false conclusions. Nearly

Table 2 Proteins related to liver metastasis formation and their function, and their differential expression when comparing primary tumors and liver metastasis by immunohistochemical technique

Proteins related to liver metastasis formation	Function	Liver expression compared to primary tumor (IHC)
E-Cadherin	Adhesion	Down-regulated[34]
Epithelial Cell Adhesion Molecule (EpCAM)	Adhesion	NA
P-Selectin and L-Selectin	Adhesion	NA
Carcinoembryonic Antigen (CEA)	Adhesion	NA
Integrin αvβ5	Adhesion, Survival	NA
sLex and sLea	Adhesion	Up-regulated[48,51]
Osteopontin (OPN)	Adhesion, Survival, Motility	Up-regulated[63]
Intracellular Adhesion Molecule (ICAM-1)	Adhesion	NA
Vascular Cell Adhesion Molecule (VCAM-1)	Adhesion	NA
CD44v6	Adhesion	NA
Cathepsine B	Invasion	NA
MMP-7	Invasion	Up-regulated[81]
MMP-2 and MMP-9	Invasion	Up-regulated[86]
Angiopoietin	Angiogenesis	Up-regulated[110]
Epidermal Growth Factor Receptor	Growth	Equal[125]
Urokinase Plasminogen Activator Receptor (uPAR)	Invasion, Motility, Dormancy	NA
Vascular endotelial Growth Factor (VEGF)	Angiogenesis	Equal[109]
Thrombospondin-1 (TSP-1)	Angiogenesis	NA
Angiostatin	Angiogenesis	NA
Endostatin	Angiogenesis	NA
Thimidine Phosphorylase (dThdPase or PDECGF)	Angiogenesis	NA
c-erb-2	Growth	NA
c-Src/β-Arrestin 1	Growth	NA
FAS Receptor (CD95)	Apoptosis	Down-regulated[134]
TRAIL Receptors (-R1, -R2, -R3 and -R4)	Apoptosis	NA
Nm23-H1 and Nm23-H2	Metastasis Suppresor Genes	NA
PRL-3	Motility, Extravasation	Up-regulated[157]

NA: Not available.

all studies lack internal and external validation tests for the generated lists of implicated genes. Different selection algorithms should be tested in order to improve the accuracy of the classifier sets[10].

In conclusion, to obtain a genetic signature for liver metastases in CRC, measures to improve reproducibility, increase consistency, and validate results need to be implemented.

Genes involved in liver metastasis formation in CRC

Alternatives to expression profiling by microarray technology have also been used in recent past years. Many genes codifying for proteins directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have been linked to mechanisms of liver metastasis in CRC[28] (Table 2).

Adhesion: Different proteins involved in adhesion/deadhesion processes have been linked to liver metastasis development in CRC. Deadhesion is a necessary step for tumor cells to detach from a tumor and disseminate. Adhesion is needed for circulating cells to contact helping counterparts in the dissemination process. It is also needed to attach to the vascular endothelium, induce endothelial retraction, and subsequently bind to glycoproteins of the basement membrane to extravasate.

E-cadherin/α-catenin is a cell to cell adhesion complex that keeps tumor cells together. Cells detaching from the primary CRC undergo an epithelial to mesenchymal transition, during which E-cadherin downregulatates in favour of other cadherins, such as N-cadherin. This process is known as the "cadherin switch" and leads to acquisition of a mesenchymal phenotype that favours invasion and migration through the stroma and thus dissemination of tumor cells[29]. Downregulation of E-cadherin/α-catenin expression has been related to tumor aggressiveness[30,31] and metastatic potential[32,33] in gastrointestinal cancers. Low expression of α-catenin and E-cadherin in CRC patients has been associated with an increment of β-catenin[34-36], advanced stages[33,37,38] and acquisition of metastatic potential[39,40]. Immunohistochemical studies show that CRCs metastasizing to liver have a significant ($P = 0.014$) reduction or complete absence of E-cadherin expression when compared to non-liver metastases[34].

Epithelial cell adhesion marker (EpCAM) is a widely expressed adhesion molecule. It has been found to present a more diffuse pattern and higher expression in CRC compared to non-malignant tissues[41]. EpCAM plays a role in modulating cadherin mediated cell-cell interactions[42] and its expression has been linked to downregulation of cadherin levels[43], suggesting that this protein possibly plays a role in ETM processes, facilitating migration and dissemination of tumor cells. Supporting this notion, isolation of EpCAM positive cells in blood samples of advanced CRC patients[44] has recently been achieved. All these preliminary data suggest that possibly EpCAM plays a role in CRC cell dissemination. Whether there is liver specificity remains unknown.

Sialyl Lewis X (sLex or CD15s) and A (sLea) are oligosaccharides commonly found in surface glycoproteins

of metastatic tumor cells[45]. sLex and sLea are natural ligands for E-selectin, which is a receptor that has been found to be expressed by activated endothelial cells. Interaction between sLex and sLea induces endothelial adhesion of tumor cells and thus favours stasis, extravasation and metastases formation. sLex and sLea expression in primary CRC have been related to poor prognosis[46] and metastatic potential[46-48] in CRC patients. sLex and sLea stain significantly positive in vessel invasion CRC cells that develop metastases compared to those that do not (71.4% *vs* 31%)[49]. sLex and sLea have been found to be present on the surface of tumor cells[50] in CRC patients who develop liver metastases. Similarly, CRC liver metastases express sLex and sLea in a larger proportion of tumor cells than in primary tumors[48,51]. E-selectin is overexpressed by endothelial cells from tumor and non-tumor vessels in CRC patients who develop liver metastasis[52,53]. In general, as has been demonstrated in *in vivo* models, glycosylated and sialylated mucins are associated with liver metastasis formation[54]. Some proteins allow the adhesion of CRC cells with blood components, such as platelets and leukocytes. Among those proteins are P-Selectin and L-Selectin. This interaction facilitates tumor emboli formation, favouring protection of tumor cells from immune attack and also enhancing their ability to contact blood vessels by mechanical means. This interaction between tumor cells and blood cells also increases contact with the endothelial surface, facilitating stasis and thus enhancing the chances of extravasation[55].

Carcinoembryonic antigen (CEA) is a cell surface glycoprotein containing significant amounts of sLex and sLea. Expression of (CEA) has been clearly correlated to generation of liver metastases in experiments transfecting CEA to CRC cell lines or administering CEA in animal models previous to CRC cell injection[56]. Initially it was speculated that CEA would act as an adhesion molecule, facilitating tumor cell aggregation and interaction with the endothelial surface. However, studies with immunosuppressed mice show that administration of intravenous CEA results in an increase of hepatic colonization and retention of CRC cells, but not an increase of adhesion[57]. Kupffer cells that express a CEA receptor bind to and degrade it, activating a signaling cascade that ends up releasing IL-1, 6 and TNF-α which, in turn, facilitates CRC cell stasis and growth[58,59]. The ability to secrete CEA offers CRC cells a selective advantage in forming metastases in the liver.

Integrins are molecules that can bind to many ECM components, such as laminin, collagen, fibronectin and vitronectin. Cancer cells expressing integrins are more likely to adhere to ECM components surrounding microvasculature. High expression of $\alpha 6\beta 4$ and $\alpha 5\beta 3$ integrins has been related to a more aggressive CRC phenotype[60,61]. Intravital fluorescence-video microscopy has been used to investigate liver metastas formation by CRC cells in animal models[62] and results have shown that $\alpha v\beta 5$ integrin is useful as an adhesion molecule and its inhibition diminished liver metastas formation.

Osteopontin (OPN) is a secreted phosphoglycoprotein capable of binding and inducing integrin-mediated cell survival, motility and anti-apoptotic intracellular pathways. OPN has been isolated in gene expression profiling studies as a candidate marker for CRC progression[20]. CRC liver metastases express OPN at higher ratios than primary CRC or normal mucosa[63]. OPN up-regulation can occur due to TCF4/LEF transcription factor activation[64]. Mechanisms by which OPN promotes liver metastases formation in CRC are unknown, but could be related to up-regulation of Upa[65], c-Met receptor and integrins[66].

Other adhesion molecules, such as the intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), have been measured in ACRC patients showing higher serum levels when compared to non-advanced CRC or healthy controls[67,68]. Nevertheless, neither clinical nor physiological relation has been established with specific development of liver metastases.

CD44 glycoprotein, more specifically v6 and v8-10 splicing variants, have been related to metastases and disease recurrence in CRC[69,70]. There is quite a bit of controversy regarding the real value of CD44 in liver metastases formation because plasma levels have not been linked to advanced stages of the disease[71] and immunohistochemical studies measuring CD44v6 staining have not found significant differences when comparing CRCs metastasizing to liver or not[34].

Invasion: Invasion processes are crucial for liver metastasis formation in CRC. Invasion occurs mainly due to basal membrane and extracellular matrix (ECM) degradation in both intravasation and extravasation steps. Some of the enzymes responsible for degradation are proteases. Among proteases, matrix metalloproteases (MMPs), cathepsins and plasminogen activators are the most relevant.

Matrylysin (MMP-7) is a proteolytic enzyme belonging to the MMPs family[72,73]. It is synthesized and secreted by tumor epithelial cells as a 28-kDa proenzyme, which can be activated through proteolytic removal of a 9-kDa prodomain from the N-terminus. The soluble activated form binds to the tumor epithelial cell surface. Both active forms, the soluble and the membrane-bound, have proteolytic activity. Its expression can be regulated by epidermal growth factor through transcription factors such as PEA3[74] or AP-1 and the β-catenin/ tcf4 complex. By degrading elastin, laminin, proteoglycans, osteopontin, fibronectin and type Ⅳ collagen, MMP-7 gains the capacity to invade. Matrilysin can also promote tumor invasion by activating other MMPs (MMP-2, MMP-9), through ectodomain shedding of E-cadherin[75] and receptor activator of nuclear factor-kappa B ligand (RANKL)[76] or through cleavage of adhesion molecules, such as integrin $\beta 4$[77].

Matrilysin has been found overexpressed in CRC[78]. MMP-7 overexpression in localized CRC disease has been correlated with invasion and liver metastasis formation[79,80]. Colorectal liver metastases show intense expression of MMP-7 compared to normal liver, and differences are more evident when comparing the MMP-7 activated form, measured by zymography, emphasizing the role of MMP-7 in CRC liver metastases formation[81]. While testing liver metastasis formation *in vivo*, it has been shown that treating colorectal cancer cells with MMP-7 specific antisense

oligonucleotides leads to a decrease in liver metastasis generation[82], while adding active MMP-7 results in an increase of liver metastasis generation[83].

MMP-9 and MMP-2 also seem to play a role in liver metastasis formation in CRC. High MMP-9 and MMP-2 levels have been detected by immunohistochemistry in the tumor-stroma interface in both primary CRC and liver metastases[84,85]. Moreover, MMP-2 and -9 activities seem to be higher in metastases than in the originating primary tumor[86]. A close correlation between high MMP-9 RNA levels and worse survival and higher risk of liver relapse after surgery has also been established[81].

Cathepsins have also been implicated in liver metastasis formation in CRC. They are a family of proteolytic enzymes with a wide variety of physiological functions. They act as serin-proteases, cystein-proteases or aspartate-proteases. They are stored as proforms in cell lysosomes and secreted to the ECM secondarily to inflammatory and oncogenic stimuli[87].

Cathepsins B, L and D are especially involved in ECM degradation in CRC. Their levels and activity[87-88] have been found to be elevated in the invasion edge of CRC. Still, Cathepsin B is the most valuable in determining invasion in CRC[89]. Cathepsin B degrades ECM directly or indirectly, by stimulating other proteases or blocking their inhibitors[87]. It can be detected in early stages of CRC but it is a good marker to determine metastatic disease[90,91]. High plasma and urine levels of Cathepsin B have been found in CRC patients[92]. *In vivo* experiments show that inhibition of Cathepsin B by selective compounds results in reduction of liver metastases formation up to 60% and reduction of liver metastases burden up to 80%[93]. A proteolytic profile, taking into account MMP and cathepsin expression, has been defined for CRC by some authors[94].

Urokinase plasminogen activator receptor (uPAR) is a factor involved in metastasis development in several cancers[95,96]. uPAR binding to urokinase plasminogen activator (uPA) enhances plasmin production which, in turn, degrades ECM and activates pro-MMPs. Inhibition of uPAR expression is associated with decreased motility and invasiveness in the human CRC cell line HCT116[97]. High uPAR expression in CRC has been related to low 5-year survival[98]. Use of antisense uPAR mRNA in a nude mice model inhibited CRC liver metastasis development[99].

During invasion, apart from basal membrane and ECM degradation processes, cancer cells have to migrate through the stroma. Clues for success are acquisition of a mesenchymal phenotype during ETM and ability to survive independently of the tumor cell population. To gain the ability to disseminate, tumor cells have to detach from the tumor population, overcome anoikis and transit from an epithelial to a mesenchymal phenotype. As a principle, cells need to be in contact with other cells in order to survive. If they lose contact or penetrate to the ECM they undergo anoikis. Overcoming anoikis, an apoptotic program related to tumor cell population detachment, is a necessary requirement to disseminate. Integrins are responsible for epithelial cancer cell cross-talk with the ECM in order to overcome anoikis, survive and migrate.

In vitro experiments have shown that activation of Src and Akt pathways are linked to decreased sensitivity of detached CRC cells to anoikis[100]. Down-regulation of $\alpha v\beta 3$ integrin has also been linked to resistance to anoikis in CRC cells[101,102]. Integrins can bind to many ECM components such as laminin, collagen, fibronectin and vitronectin. Cancer cells expressing these integrins are more likely to invade and migrate through the ECM[103,104]. High expression of $\alpha 6\beta 4$ and $\alpha 5\beta 3$ integrins has been related to more aggressive CRC phenotypes[60,61]. Intravital fluorescence-video microscopy has been used to investigate liver metastasis formation by CRC cells in animal models[62] showing that $\alpha v\beta$-integrin inhibition did not affect migration within the liver parenchyma. The role of integrins in the migration and invasion through the ECM in order to generate liver metastasis has not been extensively explored.

Angiogenesis: Different angiogenic factors have been related to metastasis formation because they can promote primary tumor growth and increase tumor cell chances to contact blood and thus disseminate. However, it is likely that angiogenesis plays a major role in metastasis generation regulating micrometastases outgrowth. Balance between angiogenic/antiangiogenic factors in the microenvironment of the metastatic tissue can promote metastasis formation by directly stimulating tumor cell growth or by increasing blood vessel formation and supply. Even in quiescent tumor cells, alteration of angiogenic balance can induce metastasis formation. This phenomenon is known as "angiogenic switch"[105] and causal factors are still under investigation.

Expression levels of vascular endothelial growth factor (VEGF) in primary CRC have been related to a poor prognosis[106]. VEGF isoform patterns have been defined using reverse transcription polymerase chain reaction (RT-PCR) analysis in 61 primary CRC. Patients developing liver metastases showed expression of VEGF121 + VEGF165 + VEGF189 at a significantly higher incidence (12 of 16, 75%) than those without liver metastasis (20 of 45, 44%) ($P = 0.036$)[107]. VEGF expression in primary CRC seems clearly associated with increased chances of dissemination. However, other studies support the contrary[108]. When VEGF mRNA levels were measured in 31 pairs of primary CRC and corresponding liver metastases, no significant differences were detected (median value 3.79 *vs* 3.97: $P = 0.989$). On an individual basis, there was a significant correlation in VEGF mRNA expression between primary CRCs and matched liver metastases ($r = 0.6627$, $P < 0.0001$). VEGF mRNA levels of patients having two or more liver metastatic tumors were significantly higher than those of patients who had solitary liver metastatic tumors in both primary cancer (5.02 *vs* 3.34: $P = 0.0483$) and liver metastases (4.38 *vs* 3.25: $P = 0.0358$)[109]. Together these results indicate that VEGF is probably not more active in metastases than in primary tumors. Despite that, increased blood supply and tumor vessel formation, as estimators of angiogenic activity, have been found to be higher in liver metastases that in primary CRC. Some molecular mediators have been thought to fulfill this role, such as angiopoietin-2 (Ang-2)[110].

Other distinctive molecules related to angiogenesis and

liver metastatic progression are platelet-derived endothelial cell growth factor or thymidine phosphorylase (PD ECGF or dThdPase). Inhibitors of angiogenesis, such as angiostatin, endostatin and thrombospondin-1 (TSP-1), either secreted by the primary or the metastatic CRC cells, can also regulate liver metastasis growth. Frequency of hepatic recurrence was significantly higher in patients with TSP-1-negative primary CRC[111]. Angiostatin transfected cells developed liver metastases in lower proportion than controls in animal models[112]. Removal of primary CRC resulted in an increase in metabolic activity in liver metastasis, while decreases in plasma levels of angiostatin and endostatin were observed. This finding indicates that primary tumors suppressed angiogenesis in distant metastases, and that removal of the primary lesion caused a flare-up in vessel neoformation and, thus, enhanced metabolic activity in liver metastases[113].

Other molecules mentioned above also contribute to liver metastasis formation through angiogenesis regulation. MMP-7 induces a direct proliferative effect on vascular endothelial cells[114] and produces angiogenesis inhibitors (angiostatin, endostatin, neostatin-7)[115] and activators (sVEGF)[116]. MMP-2 and MMP-9 stimulate degradation of ECM, increasing the availability of angiogenic activators. E-selectin acts by facilitating endothelial cell migration. α and β integrins play an important role by sending survival signals for endothelial cell maintenance[117].

Cell growth: Once established in the liver tissue microenvironment, micrometastases need growth factor stimuli in order to grow. Degradation of ECM results in an increased availability of growth and inhibitory factors. The resulting balance will then determine micrometastasic growth. Extrapolation to a non-physiological situation can be highly illustrative. Liver tissue thermal ablation was performed in mice models bearing CRC liver metastases. After ablation, increased expression of FGF-2 and VEGF was detected in the surrounding tissue. Subsequently, a greater amount of metastases occupied the regenerated thermal-ablated lobe compared with controls (55% ± 4% *vs* 29% ± 3%, $P < 0.04$)[118].

Tumor cells growth factor receptors also seem to determine success in metastatic liver growth. Her-2/neu has been detected by immunohistochemistry in 5% to 50% of primary CRC[119]. The mechanism of overexpression seems to be not linked to gene amplification. Her-2/neu positive CRCs were associated with higher postoperative non-liver specific recurrence rates (39.3% *vs* 14.6%, $P = 0.013$) and worse prognosis at 5 years (55.1% *vs* 78.3%)[120]. Other studies showed that primary CRC with high c-erbB-2 expression (27%), determined by immunohistochemical techniques, develop liver metastases more often than CRC with low c-erb-2 expression (3%)[49].

Epidermal growth factor receptors (EGFR) have been reported to be highly expressed and/or gene amplificated in 72% to 82% of metastatic CRC tissue samples[121-123]. Some studies have reported that expression of EGF receptors in CRC is associated with aggressiveness and metastatic ability. EGFR status has been shown to express similarly when measured in primary CRC and corresponding liver metastases[124]. However, some authors have seen that its status in the corresponding metastatic site is not always the same[125,126]. Conventional immunohistochemistry techniques have not been able to reveal any association between EGFR expression and outcome predicted by the biological role of EGFR in tumor behavior[127].

The C-Src gene, codifying for pp60 tyrosine kinase, has been reported to be mutated and thus is highly activated in CRC, implying an increase in proliferative potential. High activation is present especially in those CRC that metastasize to liver[128,129]. Prostaglandin E_2 (PGE_2)-induced transactivation of the EGF receptor (EGFR) in colorectal carcinoma cells has been recently found to be mediated by β-arrestin 1, which acts as an important mediator in G protein-coupled receptor-induced activation of c-Src. Interaction of beta-arrestin 1 and c-Src seems to be critical for the regulation of CRC metastatic spread of disease to the liver *in vivo*[130].

Cell survival: CRC cells need molecular factors, specifically growth factors, in order to survive in the liver parenchyma. However, there is also the need to survive immune system action (immunoescape) and to overcome anoikis.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is known to be expressed in human hepatic NK cells[131]. CRC cells expressing TRAIL-receptor would undergo apoptosis upon triggering the ligand. The same would happen in CRC cells expressing tumor necrosis factor receptor FAS (Apo-1; CD95) when contacting its corresponding ligand FASL (Apo-1L; CD95L) expressing cells, as activated lymphocytes.

During the CRC tumorigenic process, cells tend to down-regulate FAS receptor expression and up-regulate FASL[132]. Fas expression is significantly down-regulated in liver metastasis compared to corresponding primary colorectal carcinoma[133]. The link between functional Fas status and malignant phenotype was investigated using matched pairs of naturally occurring primary (Fas-sensitive) and metastatic (Fas-resistant) human colon carcinoma cell lines in both *in vitro* and *in vivo* (xenograft) settings. Results showed that loss of Fas function was linked to the acquisition of a detectable metastatic phenotype, however, only loss of Fas function was insufficient. Also, results showed that metastatic subpopulations pre-existed within the heterogeneous primary tumor and that anti-Fas interactions served as selective pressure for their outgrowth. Thus, Fas-based interactions may represent novel mechanisms for the biological or immunological selection of certain types of Fas-resistant neoplastic clones with enhanced metastatic ability[134]. Moreover, univariate and multivariate analyses revealed that Fas/CD95 expression in CRC resected liver metastases is a significant prognostic indicator of survival[135]. Increases in TRAIL sensitivity, due to changes in the balance between TRAIL receptors TRAIL-R1 and -R2 and "decoy" receptors TRAIL-R3 and -R4, have also been described during malignant progression in CRC. Still, studies measuring receptors by flow cytometry have not

been conclusive[136].

Experimental metastases studies with a CRC cell line allowed the characterization of metastatic derivatives, showing that they were less susceptible for killing by syngeneic NK cells, due to a decreased sensitivity towards TRAIL- and CD95L[137]. Data suggest that CRC cells forming metastases acquire the ability to surpass immune surveillance through desensitization to FAS/TRAIL killing. As discussed previously, integrins and Src activation may contribute to CRC progression and liver metastasis, in part, by activating survival pathways that decrease sensitivity of detached cells to anoikis[100].

Other molecules related to liver metastatic spreading: k-ras (12p) activation, present in 40% to 50% of sporadic CRC[4], has been related to a decrease in overall survival and disease free survival in CRC[6,138,139]. p53 (17p) abolition, occurring in 70% to 80% of CRC[4] and resulting in accumulation of abnormal protein detectable by immunohistochemistry, has been linked to a poor prognosis[6,140-142]. The deletion or mutation of the DCC (deleted in colorectal cancer) gene has also been related to poor prognosis tumors[143-146]. Even p53, Ras and/or DCC alterations have been linked to metastatic spreading in CRC, however, there is still no evidence specifically relating them to liver metastasis formation. The human nm23 genes, nm23-H1 and nm23-H2, are candidate metastasis suppressor genes. Their role in CRC is still confusing. Some authors claim that a reduced protein expression, secondary to gene alterations, is associated with metastasis development[147,148]. Genetic alterations were detected in four of eight CRCs associated with metastasis in lymph nodes, lung, or liver, while no alteration was observed in 12 additional CRC specimens without metastasis[149]. Others have found that gene overexpression is linked to higher recurrences, liver metastasis and decreased overall survival[150,151]. This contradiction could be explained if overexpression of nm23 was a reflection of a deletion in the nm23 gene, leading to accumulation of an altered protein product. However, more recent works have not been able to relate nm23 expression to prognosis[152-154]. The PRL-3 protein tyrosine phosphatase gene gained importance in 2001 when an article was published in Science showing that it was expressed at high levels in each of 18 cancer metastases studied but was expressed at lower levels in nonmetastatic tumors and in normal colorectal epithelium[155]. Subsequently, new data established an unexpected and unprecedented specificity in metastatic gene expression profiles: PRL-3 was apparently expressed in CRC metastasis to any organ but was not expressed in metastases of other cancers to the same organs or in nonmetastatic CRC[156]. At that time PRL-3 was determined to be a potential marker for liver metastasis of CRC with a negative impact in prognosis[157]. CRC specificity was objected to in further studies. Some authors claimed that PRL-3 acted by enhancing cell motility and thus facilitating extravasation into liver tissue[158]. The mechanism of action is still under investigation but it has already been related to integrin $\alpha 1$[159] and the Rho family of small GTPases[160].

CONCLUSION

A significant amount of experimental data points to tumor cells having a metastatic signature. This signature codifies not only for the ability to form metasteses but also for organ-specificity. DNA microarray technology has significantly improved efficiency in wide-range analysis of gene expression. Many authors have provided gene expression profiles that have been related to CRC liver metastases, however, in order to obtain a real genetic signature for liver metastases in CRC by transcription profiling, measures to improve reproducibility, increase consistency, and validate results need to be implemented. Seeking metastatic signatures through expression profiling is a tool to fight cancer, but its indiscriminate use can be misleading. Advances in molecular assays on isolated cells and in the study of cell-cell and cell-stroma interactions will likely enable the dissection of the metastatic cascade. Genes codifying for proteins directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have already been linked to mechanisms of liver metastases in CRC. Improvement in knowledge of the molecular pathways involved in the development of colorectal liver metastasis will lead to a better approach to prevent and treat this disease.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Exploiting novel molecular targets in gastrointestinal cancers

Wen W Ma, Manuel Hidalgo

Wen W Ma, Manuel Hidalgo, Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB 1M88, Baltimore, MD 21231, United States
Correspondence to: Manuel Hidalgo, MD, PhD, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, CRB 1M88, Baltimore, MD 21231, United States. wma2@jhmi.edu
Telephone: +1-410-5023543
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Abstract

Novel molecular targets are being discovered as we learn more about the aberrant processes underlying various cancers. Efforts to translate this knowledge are starting to impact on the care of patients with gastrointestinal cancers. The epidermal growth factor receptor (EGFR) pathway and angiogenesis have been targeted successfully in colorectal cancer with cetuximab, panitunumab and bevacizumab. Similarly, EGFR-targeting with erlotinib yielded significant survival benefit in pancreatic cancer when combined with gemcitabine. The multi-targeting approach with sorafenib has made it the first agent to achieve significant survival benefit in hepatocellular carcinoma. Efforts to exploit the dysregulated Akt/mTOR pathway in GI cancer therapy are ongoing. These molecular targets can be disrupted by various approaches, including the use of monoclonal antibody to intercept extracellular ligands and disrupt receptor-ligand binding, and small molecule inhibitors that interrupt the activation of intracellular kinases.

Key words: Colorectal; Pancreatic; Liver cancers; Targeted therapy; Epidermal growth factor receptor; mTOR; Angiogenesis

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INTRODUCTION

Cellular proliferation, differentiation and death are regulated by a number of extracellular factors, such as hormones, cytokines and growth hormones. Interactions between extracellular stimuli and the nucleus is mediated by a complex and interconnecting network of signaling pathways[1]. This process is often abnormal in cancer cells and our understanding of these molecular events led to the identification of novel targets for therapy development. Various approaches are been used to target these dysfunctional elements, including ligand neutralization, disruption of receptor binding, and inhibition of receptor kinases and intracellular signal messengers.

A plethora of compounds are now under development that targets these aberrant processes. Almost all of these biological agents have limited single agent activity but are synergistic when combined with conventional cytotoxic agents[2]. Therefore, they are usually tested in combination with standard therapy in specific cancer types. In colorectal cancers, fluorouracil-based regimens form the backbone of therapy in both adjuvant and metastatic settings[3-5]. Likewise, gemcitabine based therapy remains the cornerstone for untreated advanced pancreatic cancer and sorafenib is likely to become the standard therapy for hepatocellular carcinoma (HCC)[6-8].

Successful targeting of angiogenesis and the epidermal growth factor pathway has made colorectal cancer a prototypical model for the development of signaling pathway-specific agents in gastrointestinal (GI) cancers[9-11]. Akt/mTOR pathway is another candidate target in anti-cancer therapies[12]. This paper will review the approaches currently used to exploit these novel targets in the development of GI cancer therapy. The review will focus specifically on colorectal, pancreatic and primary liver cancers (hepatocellular carcinoma, or HCC).

EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY

Epidermal growth factor receptor (EGFR) is a member of the HER-family kinases, which includes EGFR, HER2, Erbb3 and Erbb4[13,14]. Upon ligand binding, EGFR homodimerizes with another EGFR or other members of the HER-family (heterodimerization), and lead to the activation of proliferative and survival signaling pathways, such as the Ras/Raf/MEK (mitogen-activated protein kinase, or MAPK) and Akt/mTOR cascades[15].

Abnormal expression or regulation of epidermal growth factors (EGF) and the receptors are implicated in the pathogenesis of many malignancies[16]. EGFR is overexpressed or up-regulated in colorectal cancers and pancreatic cancers, and is associated with early progression

and poor survival[17-22]. Similarly, EGFR is overexpressed in HCC and is associated with aggressive features with increased cellular proliferation and reduced apoptosis. *In vitro* inhibition of EGFR in HCC cell lines results in cell cycle arrest and apoptosis[23-25]. These led to the clinical development of anti-EGFR agents as single agent, or in combination therapy in view of their *in vitro* and *in vivo* synergistic activity with cytotoxic agents[26].

Cetuximab

Cetuximab is a chimeric murine/human IgG1 monoclonal antibody that blocks ligand-dependant EGFR receptor activation. The antibody has a higher affinity for the receptor than the ligands, such as EGF and transformaing growth factor (TGF-α)[27-29]. The drug is cytostatic when administered alone but highly synergistic with irinotecan in refractory colorectal cancer xenografts, leading to clinical development in irinotecan-refractory colorectal cancer patients[30,31]. In the pivotal multi-center randomized phase Ⅲ trial, 329 patients with metastatic colorectal cancer who progressed on irinotecan-based therapy were randomized to receive cetuximab alone or a combination of cetuximab and irinotecan[9]. The patients in the combination arm achieved a superior response rate of 22.9% and median time to progression of 4.1 mo compared to 10.8% and 1.5 mo in the monotherapy arm respectively. The median survival was not statistically different between the two groups.

Compared to best supportive care, metastatic colorectal cancer patients who failed multiple previous regimens achieved better overall survival, time to progression and quality of life with cetuximab monotherapy in the recent study by National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG) and Australasian Gastro-Intestinal Trials Group (AGITG)[32]. In the first line setting, Cetuximab improved response rate and time to progression when administered in combination with irinotecan-based regimen (FOLFIRI) in the CRYSTAL trial[33].

The efficacy of cetuximab with oxaliplatin-based regimen (such as FOLFOX) in second- and first-line settings is being evaluated in randomized trials (the EXPLORE and OPUS trials, respectively)[34-36]. However, the addition of cetuximab to oxaliplatin based fluoropyrimidine regimens (FOLFOX or CapOx) seemed to increase the frequency of grade 3/4 adverse events, specifically gastrointestinal toxicities, rash and lethargy[37]. The role of cetuximab in adjuvant, or postoperative, setting is being studied in 2 ongoing randomized trials (PETACC-8, Intergroup 0147) in combination with oxaliplatin-containing regimens[38-40].

Cetuximab is approved by FDA in U.S. for use in patients with EGFR-expressing colorectal cancer who failed previous irinotecan-based therapy. This was due to the fact that the trials mentioned enrolled only patients with EGFR-expressing tumors, based on preclinical data suggesting the predictive value of EGFR expression for cetuximab efficacy. However, patients with EGFR-negative colorectal cancer were later found to benefit from cetuximab therapy as well, suggesting that EGFR expression level does not correlate with cetuximab response[41,42]. This is an important lesson for the development of biological agents: patient selection based on expression, or non-expression, of specific molecular markers can be faulty. Such hypothesis should be validated vigorously in well-designed clinical trials.

The side effects of cetuximab are fairly tolerable with appropriate management. Hypersensitive infusion reaction was reported in about 3% of the patients. About 75% of patients receiving cetuximab developed a mild acneiform-like rash. The development of cetuximab-related rash seemed to correlate with response but this needs to be studied further[43].

Cetuximab was evaluated in combination with gemcitabine in advanced pancreratic cancer. Despite encouraging phase Ⅱ results, the recent randomized phase Ⅲ trial (SWOG S0205) failed to confirm the superiority of cetuximab plus gemcitabine combination over gemcitabine monotherapy in this patient population[44].

Cetuximab monotherapy proved to be tolerable in patients with advanced HCC though activity was lacking in phase Ⅱ trials[27,45]. Gruenwald *et al* enrolled 32 unresectable HCC patients and 27 were evaluable. Seventy-two percent (23 of 32) had Child-Pugh Stage A cirrhosis, 25% Stage B and 3% Stage C. Previously treated patients were eligible for this trial and 44% achieved stable disease for at least 8 wk and median time to progression was 22.5 wk. The agent is been evaluated in combination with cytotoxic chemotherapy in HCC[46].

Panitumumab

Panitumumab is a fully humanized anti-EGFR monoclonal antibody that is being evaluated in metastatic colorectal cancer. The agent has the advantage of avoiding the hypersensitive reaction typical of chimeric murine proteins, such as cetuximab. In a multi-institutional phase Ⅲ trial, patients with refractory metastatic colorectal cancer were randomized to receive panitumumab plus best supportive care or best supportive care alone[47]. Eight percent (8%) of patients receiving panitumumab achieved partial response. About 90% developed the characteristic acneiform rash comparable to cetuximab monotherapy. As expected and importantly, hypersensitivity infusion reaction for the humanized monoclonal antibody was lower than that reported for cetuximab. Combination regimens containing panitunumab are been evaluated clinically.

Erlotinib

Erlotinib is an oral quinazoline that reversibly inhibits EGF receptor tyrosine kinase. The small molecule induces in vitro cell cycle arrest and apoptosis, and has *in vivo* anti-tumor effects[48,49]. Major side effects are rash and diarrhea, characteristic of this class of drug. Erlotinib was approved in 2004 by FDA in U.S. for use as single agent in previously treated non-small cell lung cancer (NSCLC) following the demonstration of survival benefit in a randomized phase Ⅲ trial (NCIC-CTG BR.21)[50]. EGFR mutations seems to correlate with the efficacy of anti-EGFR therapy in NSCLC though effort to uncover additional molecular predictors continues[51].

Among GI cancers, erlotinib is furthest along clinical development in pancreatic cancer. Gemcitabine has been

Table 1 Agents targeting EGFR pathway in GI cancers

Agents	Tumor types	Regimen	Study design	References
Monoclonal antibodies				
Cetuximab	Colorectal cancer	Irinotecan/cetuximab	Phase Ⅲ	[9]
	Hepatocellular carcinoma	Cetuximab	Phase Ⅱ	[27]
	Pancreatic cancer	Gemcitabine/cetuximab	Phase Ⅱ	[28]
	Pancreatic cancer	Gemcitabine/RT/cetuximab	Phase Ⅱ	[29]
Panitumumab	Colorectal carcinoma	Panitumumab	Phase Ⅲ	[47]
Matuzumab	Pancreatic cancer	Gemcitabine/matuzumab	Phase Ⅰ	[60]
	Colorectal cancer	Matuzumab	Phase Ⅰ	[61]
Tyrosine kinase inhibitors				
Erlotinib	Pancreatic cancer	Gemcitabine/erlotinib	Phase Ⅲ	[52]
	Colorectal cancer	CapOx/erlotinib	Phase Ⅱ	[54]
	Hepatocellular carcinoma	Erlotinib	Phase Ⅱ	[56]
	Colorectal cancer	FOLFIRI/erlotinib	Phase Ⅰ	[55]
	Pancreatic cancer	Gemcitabine/paclitaxol/RT/erlotinib	Phase Ⅰ	[62]
Geftinib	Colorectal cancer	Gefitinib/fluorouracil/oxaliplatin	Phase Ⅱ	[63]
	Colorectal cancer	Gefitinib/oxaliplatin	Phase Ⅱ	[64]
	Colorectal cancer	Gefitinib	Phase Ⅱ	[65,66]
	Hepatocellular carcinoma	Gefitinib	Phase Ⅱ	[67]
	Pancreatic and rectal cancer	Capecitabine/gefitinib/RT	Phase Ⅰ	[68]
Lapatinib	Colorectal cancer	Lapatinib	Phase Ⅱ	[59]

RT: Radiation therapy.

the standard first-line therapy for advanced pancreatic cancer in improving symptoms and survival, but not curative[6]. In the NCIC-CTG sponsored multi-institutional trial, 569 patients with untreated advanced pancreatic adenocarcinoma were randomized to receive gemcitabine plus erlotinib or gemcitabine plus placebo[52]. Intension-to-treat analysis showed longer survival in patients receiving erlotinib plus gemcitabine (6.24 mo *vs* 5.91 mo; HR 0.82, $P = 0.038$) compared to gemcitabine only. One year survival was also higher in the erlotinib-containing arm (23% *vs* 17%, $P = 0.023$). Unlike colorectal cancer, tumor EGFR expression was not a pre-requisite in this trial. There was more frequent mild grade rash, diarrhea and hematological toxicity in the combination arm but the frequency of moderate and severe toxicities were comparable in both arms. However, routine use of erlotinib and gemcitabine combination cannot be recommended in patients with advanced pancreatic cancer in view of the high cost of erlotinib[53].

Erlotinib use in colorectal cancer remains investigational. The drug showed encouraging result when used in combination with capecitabine and oxaliplatin in previously treated disease in phase Ⅱ trial[54]. The result needs to be validated in a larger randomized trial. The drug had unacceptably high rate of toxicity when combined with dose-reduced FOLFIRI in patients with metastatic colorectal cancer[55].

Erlotinib is being tested in untreated advanced HCC patients in an ongoing open-labeled phase Ⅱ trial[56]. Tumor EGFR expression is not an exclusion criteria in this trial. Interim analysis of 25 patients suggested a longer median survival among erlotinib-responding patients of 44 wk compared to 25 wk in erlotinib-non-responders. All responders developed rashes. The trial aims to accrue a total of 40 patients.

Lapatinib

Lapatinib is an interesting oral inhibitor of two tyrosine kinases: ErbB1 (EGFR) and ErbB2 (HER-2/*neu*). The agent has significant efficacy in advanced breast cancer when combined with capecitabine[57]. Both EGFR and HER-2/*neu* are co-expressed in colorectal cancer cells and simultaneous targeting of these receptors in preclinical studies enhanced apoptosis. Lapatinib is currently being tested in previously treated colorectal cancer patients[58,59].

EGFR pathway proves to be a valid target in GI cancers, especially in colorectal cancer with cetuximab and panitumumab. The small but statistically significant survival improvement by erlotinib in pancreatic cancer has been more a demonstration of "proof-in-principle" and the optimal approach to using anti-EGFR agents in pancreatic cancer still needs to be defined. Lapatinib development will hopefully shed light on whether dual-targeting of the ErbB receptor family is a successful approach in colorectal cancer (Table 1).

ANGIOGENESIS

Angiogenesis is vital to cellular growth, reproduction and development[69]. The process is often pathological in cancers, driven by an imbalance of pro- and anti-angiogenic factors in tumors[70]. The resulting tumor-induced vasculature is often leaky and dysfunctional, leading to increase interstitial pressure that impedes the delivery of both oxygen and chemotherapeutic agents[71].

VEGF-A (commonly known as VEGF) is among the first angiogenic factor discovered and shares sequence homology to the platelet-derived growth factor (PDGF) superfamily[72,73]. VEGF-A interacts with two transmembrane receptor tyrosine kinases: VEGFR-1 (Flt-1) and VEGFR-2 (KDE, Flk-1). VEGFR-2 is the primary mediator of VEGF-A and is often overexpressed in tumor vasculatures. Activation of VEGFR-2 promotes endothelial cell proliferation, survival and migration. As such, VEGFR-2 has been a major anti-angiogenic target.

VEGF over-expression and increased microvessel

density correlated with disease recurrence, metastases and survival in colorectal cancers[74-84]. Similarly, increased VEGF expression in pancreatic adenocarcinoma was also associated with poor prognosis though some studies suggest that PDGF and bFGF, instead of VEGF-A, are more important in the modulation of angiogenesis in pancreatic cancer[85-88]. HCC is highly vascular and patients with the liver neoplasm have higher serum VEGF levels than those with benign liver tumors[89-91]. In addition, increased VEGF expression following surgical resection or prior to transarterial chemoembolization correlated with poor prognosis[92-95].

As such, angiogenesis has been a focus of GI cancer therapy and can be accomplished by monoclonal antibody and small molecule tyrosine kinase inhibitor. These anti-angiogenic agents are believed to exert their anti-tumor effects by either affecting the tumor directly, inhibiting neovascularization, or enhancing chemotherapy delivery by normalizing the tumor vasculature[71,96].

Bevacizumab

Bevacizumab is a humanized monoclonal VEGF-binding antibody with anti-angiogenic properties that is the furthest along clinical development in its class. The drug was approved by FDA in U.S. for use with intravenous fluorouracil-containing regimens in patients with metastatic colorectal cancer[97].

The hint for bevacizumab efficacy in colorectal cancer in first-line setting was observed in a phase Ⅱ trial. 104 patients with metastatic colorectal cancer were randomized to receive fluorouracil and leucovorin (5FU/LV) (control arm), 5FU/LV plus "low dose" bevacizumab (5 mg/kg) and 5FU/LV plus "high dose" bevacizumab (10 mg/kg)[98]. Patients in both bevacizumab-containing arms achieved higher response rate (control: 17%; "low dose" bevacizumab: 40%; "high dose": 24%), longer time to progression and median survival (13.8 mo; 21.5 mo; 16.1 mo, respectively). Interestingly, outcome was better in the "low dose" bevacizumab arm than the "high dose" arm and was attributed partly to a higher proportion of poor risk patients in the "high dose" arm. Bevacizumab-related toxicities in this trial included thrombosis, hypertension, proteinuria and epistaxis. Bevacizumab at 5 mg/kg was thus chosen as the recommended dose for further development.

Bevacizumab was subsequently tested in metastatic colorectal cancer patients in combination with 5FU, leucovorin, leucovorin and irinotecan (IFL) in the pivotal phase Ⅲ trial. 813 patients with untreated metastatic colorectal cancer were randomized to receive IFL plus placebo (control arm), IFL plus bevacizumab 5 mg/kg or 5FU/LV plus bevacizumab 5 mg/kg[10]. IFL superseded 5FU/LV as the standard first-line regimen in U.S. by the time this trial was planned and was chosen as the control arm. The 5FU/LV plus bevacizumab arm was added as a backup since the safety of IFL plus bevacizumab was unknown. The 5FU/LV/bevacizumab arm was discontinued later during the planned interim analysis when IFL plus bevacizumab proved to be safe. The superior survival of 20.3 mo in the IFL plus bevacizumab over the IFL plus placebo arm of 15.6 mo supported the use of bevacizumab in the first-line treatment of metastatic colorectal cancer. Consistent with the earlier phase Ⅱ trial, reversible hypertension and proteinuria were more frequent with bevacizumab use. Other rare but serious side effects include gastrointestinal perforation, thrombosis and wound dehiscence.

Bevacizumab was also tested in metastatic colorectal cancer combined with oxaliplatin-based regimen in second-line setting. In the randomized phase Ⅲ trial (E3200), patients with previously treated colorectal cancer were randomized to 3 arms: FOLFOX4 plus bevacizumab, FOLFOX4 and bevacizumab only. The dose of bevacizumab chosen was 10 mg/kg[99]. The patients were not exposed to bevacizumab previously. Preliminary result showed superior survival and progression free survival in the FOLFOX4 plus bevacizumab arm. In a separate analysis, 56% of patients receiving FOLFOX4 plus bevacizumab had bevacizumab dose reduction but the survival was not significantly different from those without dose reduction[100]. Preliminary results indicate that bevacizumab is equally effective with oxaliplatin-based regimen and should be considered in second-line setting for metastatic colorectal cancer patients without previous bevacizumab exposure.

Despite the progress with bevacizumab in metastatic colorectal cancer therapy, many clinical questions remained unanswered, such as the role of continuing bevacizumab from first- into second-line setting and the synergism of bevacizumab with oral fluoropyrimidines. The combination of bevacizumab, erlotinib plus FOLFOX was examined in a phase Ⅱ trial but 40% of patients developed unacceptable toxicity and the treatment was stopped[101]. Bevacizumab is been tested with FOLFIRI in an ongoing phase Ⅱ trial involving patients with metastatic colorectal cancer[102].

The combination of bevacizumab and gemcitabine was been evaluated in pancreatic cancer. The multi-center phase Ⅱ trial demonstrated a modest partial response rate of 21% in untreated advanced pancreatic cancer patients treated with the combination[103]. Unfortunately, the combination failed to achieve survival improvement compared to gemcitabine only therapy in the subsequent phase Ⅲ randomized trial (CALGB 80303)[104]. The combination of bevacizumab with gemcitabine plus oxaliplatin (GemOx) is being evaluated in an ongoing North Central Cancer Treatment Group phase Ⅱ trial[105].

VEGF-Trap

VEGF-Trap (Regeneron) is a novel chimeric decoy receptor with higher affinity for VEGF-A than monoclonal antibodies[106]. The molecule consists of the extracellular domains of VEGFR-1 and -2 fused to the constant region (Fc) of IgG1[107]. Preclinical studies demonstrated potent anti-tumor and anti-angiogenic activities in various cancer models, prompting further clinical testing of the agent[108,109]. Phase I study of the agent in patients with advanced solid tumors showed that the agent is well-tolerated and the toxicities, including fatigue, pain, constipation and arthralgia, can be managed safely[110]. VEGF-Trap is being tested with fluorouracil-based regimens in phase I trials[111,112].

Table 2 Agents targeting angiogenesis in GI cancers

Agents	Tumor types	Regimen	Study Design	References
Monoclonal antibodies				
Bevacizumab	Colorectal cancer	Bevacizumab/IFL	Phase Ⅲ	[10]
		Bevacizumab/FOLFOX (E3200)	Phase Ⅲ	[99]
		Bevacizumab/FOLFIRI	Phase Ⅱ	[102]
	Pancreatic cancer	Bevacizumab/gemcitabine	Phase Ⅱ/Ⅲ	[103]
		Bevacizumab/gemcitabine/oxaliplatin	Phase Ⅱ	[105]
		Bevacizumab/capecitabine/RT	Phase Ⅰ	[124]
VEGF decoy				
VEGF-Trap	Solid tumors	I-LV5FU2/ VEGF-Trap	Phase Ⅰ	[111]
	Solid tumors	FOLFOX4/ VEGF-Trap	Phase Ⅰ	[112]
Tyrosine kinase inhibitors				
Sorafenib	Hepatocellular carcinoma	Sorafenib	Phase Ⅲ	[8]
	Pancreatic cancer	Gencitabine/sorafenib	Phase Ⅰ	[116]
	Colorectal cancer	Oxaliplatin/sorafenib	Phase Ⅰ	[115]
Sunitinib	Colorectal cancer	Irinotecan/cetuximab/sunitinib	Phase Ⅰ/Ⅱ	[122]
	Hepatocellular carcinoma	Sunitinib	Phase Ⅰ/Ⅱ	[121,123]

IFL: Irinotecan/leucovorin/bolus fluorouracil; FOLFOX: Oxaliplatin/leucovorin/infusional fluorouracil; FOLFIRI: Irinotecan/leucovorin/infusional fluorouracil; RT: Radiation therapy.

Sorafenib

Sorafenib (BAY43-9006) is an oral bi-aryl urea initially developed as a potent inhibitor of Raf protein[113]. The agent is also a multi-target kinase inhibitor and has significant activity against VEGFR-1, VEGFR-2, VEGFR-3 and PDGFR. As such, sorafenib is also been evaluated for its anti-angiogenic properties. The drug significantly inhibits neovascularization in colon, breast and non-small cell lung cancer xenografts in preclinical studies, marked by decreased tumor microvessel density.

Phase I trial involving patients with refractory solid tumors showed that sorafenib is fairly well tolerated. The main toxicities were diarrhea, skin rash and fatigue[114]. Downstream ERK protein was significantly inhibited at sorafenib $\geqslant$ 200 mg bid dose, indicating Raf inhibition. Partial response was observed in one (of 6) patients with HCC (400 mg bid dose) and stable disease for more than 6 mo in 6 (of 26) of colorectal cancer patients[115,116].

Sorafenib became the first agent to achieve significant survival benefit in advanced HCC in a multi-center randomized trial (SHAPR trial)[8]. 602 patients with previously untreated advanced disease with Child-Pugh Stage A cirrhosis and good performance status (ECOG PS 0-2) were randomized to receive sorafenib or placebo. Compared to the placebo arm, patients receiving sorafenib had a longer median survival (10.7 mo *vs* 7.9 mo; HR 0.69, $P < 0.01$) and time to progression (HR 0.58, $P < 0.01$). Serious side effects were similar in both groups though diarrhea and hand-foot syndrome were more frequent in those receiving sorafenib. Criticisms of the study include the generalisability of the result since majority of the patients enrolled were European and had minimal liver dysfunction. The benefit in Child's B and C patients remains unclear. Moreover, the therapy is quite costly and is a significant financial burden for most HCC patients who live in poorer developing countries. Sorafenib continues to be evaluated in HCC in combination therapy.

Sunitinib

Sunitinib (SU11248) is an oral inhibitor of VEGFR-2, PDGFR, c-kit and FLT-3. Preclinical studies showed anti-tumor activity in various malignancies, including leukemia, breast and lung cancer models[117-119]. In a phase I study, the recommended dose for sunitinib was determined to be 50 mg/d on a "4-wk-on/2-wk-off" schedule[120]. The toxicities include hypertension, thrombocytopenia, neutropenia, diarrhea, hair and skin changes. Sunitinib is being tested in HCC and in combination with irinotecan and cetuximab in previously treated metastatic colorectal cancer[121-123].

Of the anti-angiogenic agents discussed, bevacizumab proved to be an exceptionally efficacious agent in colorectal cancer when combined with conventional cytotoxic agents. However, this monoclonal antibody failed to achieve the clinical benefit expected in pancreatic cancer in combination therapy. More excitingly, sorafenib becomes the first chemotherapeutic agent to achieve significant clinical benefit in HCC (Table 2).

AKT/mTOR PATHWAY

The mammalian target of rapamycin (mTOR) is a cytosolic serine/threonine kinase that plays a central role in cell proliferation and survival[125]. The kinase is downstream to the phosphatidylinositol 3'-kinase (PI3K)/Akt signaling pathway. Activated mTOR interacts with downstream effectors, such as 4E-BP1 and p70s6K, to modulate various growth and survival-related cellular functions. The pathway is sensitive to extracellular growth factors (EGF, VEGF and IGF) and nutrients (amino-acids, glucose and oxygen).

In a series of 101 resected primary hepatoma (with 73 HCC), 15% had overexpression of phospho-mTOR and 5% had increased total mTOR protein expression[126]. In pancreatic cancers, more than 90% of the tumors contain an activating upstream ras mutation and about half of the surgically resected pancreatic cancer specimens had mTOR activation[127-131].

Loss of the suppressive PTEN gene expression, PI3K gene mutations and amplification of Akt result in constitutive activation of the upstream PI3K/Akt pathway

observed in some tumors[126-129,132-135]. Such activation increases the tumors' susceptibility to mTOR inhibitors and provided the rationale in developing rapamycin (mTOR inhibitor) analogs in various cancer types[136-140]. In addition, inhibition of mTOR reversed gemcitabine resistance in gemcitabine-resistant pancreatic cancer cell lines in preclinical xenograft model[131]. These preclinical data support the clinical testing of mTOR inhibitors in HCC and pancreatic cancer.

Rapamycin

Rapamycin (sirolimus) is an oral macrolide derived from *Streptomyces hygroscopicus* that is widely used as immunosuppressant in organ transplantation[141-145]. Rapamycin and its analogs also inhibit cellular proliferation in a wide range of human tumors. The drug complexes with FKBP12, a member of the immunophilin family of FK506-binding proteins, intracellularly which in turn inhibits the mTOR kinase activity, leading to G1 phase cell cycle arrest and apoptosis[146,147]. However, the drugs poor aqueous solubility, chemical stability and lack of investor interest impeded its clinical development as an anti-neoplastic agent[12]. Currently, rapamycin is being tested in a pharmacodynamic-guided dose-finding study involving patients with advanced solid tumor and also in a phase Ⅱ trial involving patients with advanced pancreatic cancer[148].

Temsirolimus

Temsirolimus (CCI-779) is a water-soluble synthetic rapamycin ester with significant anti-proliferative properties that can be administered *via* both oral and intravenous routes[149-154]. The drug demonstrated comparable *in vitro* anti-tumor effect to rapamycin against a wide range of human cancer cell lines, including prostate, breast, small-cell lung carcinoma, melanoma, glioblastoma and T-cell leukemia. The agent inhibits tumor growth, or is cytostatic, in a variety of cancer xenograft models but did not achieve tumor shrinkage.

Two dosing schedules of temsirolimus were tested in separate phase I trials: weekly intravenous dose versus the 30 minute intravenous infusion administered daily for 5 d on a bi-weekly schedule[155,156]. Toxicities observed include skin changes, muscostomatitis, asthenia, myelosuppression (thrombocytopenia, neutropenia), dyslipidemia and elevated liver enzymes. Dose escalation for the weekly regimen was stopped at 220 mg/m^2, which was the highest planned dose. Toxicities were fairly manageable and reversible at this dose. Interestingly, tumor shrinkages (partial and minor responses) were observed clinically, contrary to the cytostatic phenomenon seen in preclinical studies. Two patients achieved partial response: one with renal cell carcinoma and another with breast cancer. This led to further testing of temsirolimus in various cancer types[157-160]. Temsirolimus was recently approved by FDA in U.S. for the treatment of poor risk renal cell carcinoma patients based on the positive result from a randomized phase Ⅲ trial[161].

Everolimus

Everolimus (RAD001) is an oral rapamycin analog that inhibits tumor growth and angiogenesis in a dose-dependant manner and has anti-proliferative activity against a wide range of human cancers[162,163]. The optimal biologically active dose of everolimus was studied in two phase I trials. Everolimus 20 mg weekly was determined to be biologically active and toxicities associated with weekly everolimus administration were well tolerated and included anorexia, fatigue, rash, mucositis, headache, hyperlipidemia and gastrointestinal disturbance. The dose-limiting toxicities of daily everolimus were stomatitis, neutropenia and hyperglycemia. Pre-treatment and during-treatment tumor biopsies were done to evaluate pharmodynamic effects of everolimus and a 10 mg daily dose was recommended as the optimal dose. Partial response was seen in one colorectal cancer patient and everolimus is in phase Ⅱ development as single agent in refractory colorectal cancer[164]. The agent is being developed in other cancer types as well, such as gastrointestinal stromal tumor, neuroendocrine tumors, renal cell carcinoma, non-small cell lung cancer and melanoma[165-169].

The Akt/mTOR pathway seems to be an important survival and pro-growth pathway in GI cancers. Temsirolimus is the first of its class to achieve significant anti-tumor efficacy and clinical development of the class of mTOR inhibitors in pancreatic cancer and HCC continues.

CONCLUSION

Angiogenesis and EGFR pathways were hypothesized as targets for anticancer therapy more than three decades ago. Efforts to translate this knowledge to bedside are just starting to benefit patients with GI cancers. Successful development of cetuximab and bevacizumab in colorectal cancer ushered in the era of biologically targeted agents in the fight against GI cancers. More milestones were later achieved when the survival of previously difficult-to-treat GI cancers were improved by these novel biological agents, as in the case of erlotinib in pancreatic cancer and sorafenib in HCC. More molecular targets will become apparent as our knowledge of the complex neoplastic processes increases, and will provide exciting translational opportunities in the development of GI cancer therapy.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

New approaches in angiogenic targeting for colorectal cancer

Aleix Prat, Esther Casado, Javier Cortés

Aleix Prat, Esther Casado, Javier Cortés, Department of Medical Oncology, Vall d'Hebron University Hospital, Barcelona, Spain
Esther Casado, Department of Medical Oncology, Hospital General de Manresa, Barcelona, Spain
Correspondence to: Javier Cortés, Department of Medical Oncology, Vall d'Hebron University Hospital, Paseo Vall d' Hebron, 119-129, Barcelona 08035, Spain. jcortes@vhebron.net
Telephone: +34-934-893000 Fax: +34-932-746059
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Abstract

Colorectal carcinoma (CRC) is one of the leading causes of cancer death worldwide. In the last decade, the addition of irinotecan and oxaliplatin to standard fluorouracil-based chemotherapy regimens have set the new benchmark of survival for patients with metastatic CRC at approximately 20 mo. Despite these advances in the management of CRC, there is a strong medical need for more effective and well-tolerated therapies. The dependence of tumor growth and metastasis on blood vessels makes angiogenesis a rational target for therapy. One of the major pathways involved in this process is the vascular endothelial growth factor (VEGF) and its receptors (VEGFR). In 2004, the first agent targeting angiogenesis, bevacizumab (BV), was approved as an adjunct to first-line cytotoxic treatment of metastatic CRC. The role of BV as part of adjuvant treatment and in combination with other targeted therapies is the subject of ongoing trials. However, BV is associated with an increase in the risk of arterial thromboembolic events, hypertension and gastrointestinal perforations and its use must be cautious. Novel VEGFR TK inhibitors with different ranges of nanomolar potencies, selectivities, and pharmacokinetic properties are entering phase Ⅲ trials for the treatment of cancer. Conversely, one of these novel agents, vatalanib, has been shown not to confer survival benefit in first and second-line treatment of advanced CRC. The basis of these findings is being extensively evaluated. Ongoing and new well-designed trials will define the optimal clinical application of the actual antiangiogenic agents, and, on the other hand, intensive efforts in basic research will identify new agents with different antiangiogenic approaches for the treatment of CRC. In this review we discuss and highlight current and future approaches in angiogenic targeting for CRC.

INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of cancer death worldwide despite progressive improvements in preventive, diagnostic, and therapeutic approaches[1]. Approximately 50 percent of patients who undergo potentially curative surgery alone ultimately relapse and die of metastatic disease[2]. From the late 50 s, 5-fluorouracil (5-FU) was the only drug approved for the treatment of advanced CRC with an overall response rate (RR) and median survival of 10% and 10 mo, respectively[3,4]. This RR was improved to nearly 25% when leucovorin (LV) was used to modulate 5-FU[5]. Recently, irinotecan and oxaliplatin have been added to the armamentarium of agents with activity in CRC. The addition of these two cytotoxic agents to the standard 5-FU/LV-based regimens improves not only RR, but also overall survival (OS) over 5-FU/LV alone, setting the new benchmark of survival for patients with unresectable advanced CRC at around 20 mo[6-10]. Despite these advances in the management of CRC, there is a strong medical need for more effective and well-tolerated therapies and further improvements in survival are anticipated with the introduction of novel targeted therapies both as single agents and in combination. Among them, anti-angiogenesis agents have become a new therapeutic approach in the metastatic setting. In this review we will discuss and highlight current and future approaches in angiogenic targeting for CRC.

ANGIOGENIC TARGETING

The dependence of tumor growth and metastasis on blood vessels makes angiogenesis one of the fundamental hallmarks of cancer[11] and a rational target for[12]. Several growth factor receptor pathways have been implicated in the promotion of tumor angiogenesis. One of the major pathways involved in this process is the vascular endothelial

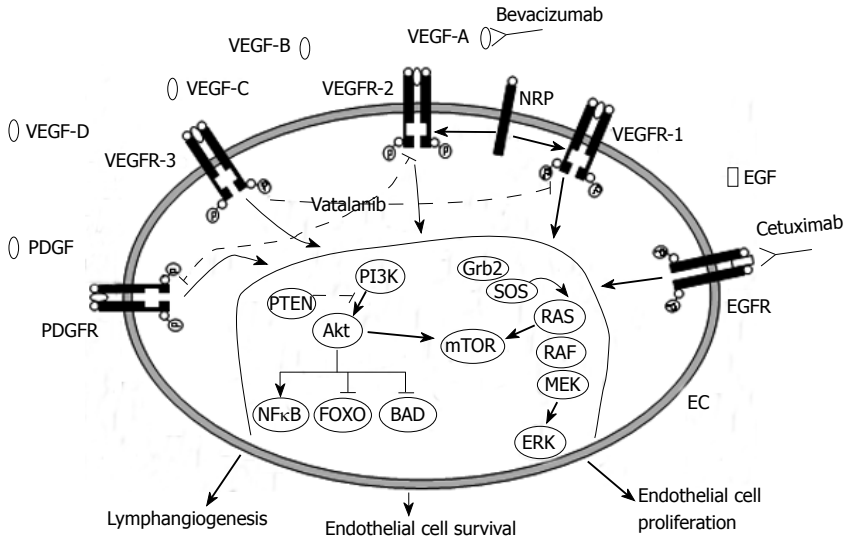

Figure 1 Vascular endothelial growth factor (VEGF) signaling network and novel targeted therapies. VEGFR: Vascular endothelial growth factor receptor; PDGF: Plateled-derived growth factor; PDGFR: Plateled-derived growth factor receptor; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; NRP: Neuropilin; EC: Endothelial cell.

growth factor (VEGF) family of proteins, also known as vascular permeability factors, and its receptors (Figure 1). The VEGF pathway plays a crucial role in normal and pathologic angiogenesis, triggering multiple signaling networks that result in endothelial cell survival, migration, mitogenesis, differentiation, and vascular permeability[13]. The VEGF-related gene family of angiogenic and lymphangiogenic growth factors comprises six secreted glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF) 1 and 2. The primary effects of VEGF ligands are mediated through binding to the VEGF tyrosine kinase receptors (VEGFR): VEGFR-1, which binds VEGF-A, VEGF-B, and PlGF-1; VEGFR-2, which binds VEGF-A, VEGF-C, VEGF-D, and VEGF-E; and VEGFR-3, which binds VEGF-C and VEGF-D, and its expression is limited to the lymphatic endothelial cells. In addition to these receptors, VEGF interacts with neuropilins, a family of activating coreceptors without an intracellular signaling domain[14,15]. VEGFR-1 and VEGFR-2 have seven extracellular immunoglobulin-like domains, a single transmembrane region and a consensus kinase sequence that is interrupted by a kinase-insert domain[16]. Once bound by VEGF, two receptors dimerize, and the tyrosine kinase domain of each receptor "autophosphorylates" the other, leading to an active receptor that initiates a signaling cascade. The VEGF pathway is upregulated by hypoxia[17] and by several growth factors, such as epidermal growth factor (EGF)[18], platelet-derived growth factors (PDGFs)[19,20], hepatocyte growth factor[21] and other cytokines.

Overexpression of VEGF has been associated with tumor progression and poor prognosis in several tumor systems, including CRC[22,23]. Preoperative serum VEGF have also been shown to correlate with advanced tumor stage or nodal status at the time of surgery[24]. Furthermore, intense expression of VEGF mRNA is detected in human liver metastases from primary colon or rectal carcinomas[25]. In 1993, Kim *et al*[26] reported that antibodies to VEGF exert a potent inhibitory effect on the growth of several tumor cell lines in nude mice. In addition, the combination of anti-VEGF antibody and chemotherapy in nude mice injected with human cancer xenografts has an increased antitumor effect compared with antibody or chemotherapy treatment alone[27]. It is, therefore, not surprising that most of the antiangiogenesis treatment strategies focus on inhibition of the VEGF pathway and its regulators. However, the mechanisms of action of anti-VEGF therapy in cancer patients are still far from being fully understood.

In December 2004 the first agent targeting angiogenesis, bevacizumab (Avastin®; Genentech, Inc., South San Francisco, CA), was approved to be given intravenously as a combination treatment along with standard chemotherapy drugs for metastatic CRC, increasing RR, progression-free survival (PFS) and overall survival (OS) with limited toxicity[28]. Gradually, many other antiangiogenic agents that target the VEGF pathway are entering the clinic. These novel targeted agents inhibit the VEGF pathway by targeting the VEGF ligand, its receptors or by blocking downstream signaling pathway components. Antiangiogenic agents include antibodies, low-molecular-weight tyrosine kinase (TK) inhibitors, antisense oligonucleotides and aptamers (Table 1).

BEVACIZUMAB IN CRC

Bevacizumab (BV) is a recombinant humanized monoclonal antibody that binds to all isoforms of VEGF-A with a reported half-life of 17-21 d[29]. In phase I trials, BV was generally well tolerated and did not demonstrate dose-limiting toxicity or interactions with commonly used chemotherapy regimens[30,31]. Based on the data obtained in these phase I trials, Kabbinavar *et al*[32] conducted a randomized, phase II trial comparing the safety and efficacy of BV (at two dose levels, 5 and 10 mg/kg every 2 wk) plus 5-FU (500 mg/m^2)/LV (500 mg/m^2) versus 5-FU/LV alone as first-line therapy for metastatic CRC(Table 2). One hundred and two patients were included. Administration of BV at low-dose and high-dose every 2 wk resulted in a significant increase of 3.8 mo and 2.0 mo, respectively, in the estimated progression-free survival (PFS) compared with 5-FU/LV alone. Treatment with 5-FU/LV/BV at both dose levels compared with 5-FU/LV resulted in

Table 1 Anti-VEGF agents currently in clinical development

Agent	Targets	Phase of development
Specific anti-VEGF antibodies		
Bevacizumab (Avastin)	VEGF-A	Phase Ⅲ
IMC-C1121b	VEGFR-2	Phase Ⅰ-Ⅱ
VEGF Trap	VEGF, PlGF, VEGF-B	Phase Ⅰ
Agents that target VEGF receptors tyrosine kinase		
Vatalanib (PTK787/ZK 222584)	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Phase Ⅲ
Sorafenib (BAY 43-9006)	VEGFR-2, PDGFR-β, FLT3, c-Kit, Raf	Phase Ⅲ
Sunitinib (SU11248)	VEGFR2, PDGFR-β, FLT3, c-Kit	Phase Ⅲ
Semaxanib (SU5416)	VEGFR1, VEGFR2	Stopped
AZD2171	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Phase Ⅰ-Ⅱ
CEP-7055	VEGFR1, VEGFR2, VEGFR3	Phase Ⅰ-Ⅱ
CHIR258	VEGFR1, VEGFR2, FGFR1, FGFR3	
CP-547632	VEGFR2	Phase Ⅰ-Ⅱ
GW786034	VEGFR2	Phase Ⅰ-Ⅱ
OSI-930	VEGFR, c-Kit	Phase Ⅰ-Ⅱ
ZK-CDK	VEGFRs, PDGFR, CDKs	Phase Ⅰ-Ⅱ
AG013736	VEGFR, PDGFR-β, c-Kit	Phase Ⅰ-Ⅱ
AMG706	VEGFR1, VEGFR2, PDGFR-β, c-Kit	Phase Ⅰ-Ⅱ
KRN-951	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Phase Ⅰ-Ⅱ
BMS-582664	VEGFR2, FGFR	Phase Ⅰ-Ⅱ
XL999	FGFR, VEGFRs, PDGFR, FLT3	Phase Ⅰ-Ⅱ
Zactima (ZD6474)	VEGFR2, EGFR, RET	Phase Ⅰ-Ⅱ
AEE788	VEGFR1, VEGFR2, EGFR	Phase Ⅰ-Ⅱ
Antisense oligonucleotides		
Veglin (VEGF-AS)	VEGF, VEGF-C, VEGF-D	Phase Ⅰ
Aptamer		
Aplidin (Dehydrodidemnin B)	VEGF	Phase Ⅰ

CDK: Cyclin-dependent kinase; EGFR: Epidermal growth factor receptor; FGFR: Fibroblast growth factor receptor; FLT3: Fms-related tyrosine kinase 3; MMP: Matrix metalloproteinase; PDGFR: Platelet-derived growth factor receptor; PIGF: Placental growth factor; VEGF: Vascular endothelial growth factor.

Table 2 Completed trials for Bevacizumab with chemotherapy in metastatic CRC

REF	Regimen	Pts	RR (%)	*P*	PFS or TTP (mo)	*P*	OS (mo)	*P*
28	IFL	411	35	0.004	6.2	< 0.001	15.6	< 0.001
	IFL + BV	402	45		10.6		20.3	
32	5-FU/LV	35	17	-	5.2	-	13.6	-
	5-FU/LV + BV-low	35	40	0.029	9	0.005	21.5	0.137
	5-FU/LV + BV-high	32	24	0.434	7.2	0.217	16.1	0.582
41	5-FU/LV	105	15	0.055	5.5	0.0002	12.9	0.16
	5-FU/LV + BV	104	26		9.2		16.6	
43	FOLFOX	289	9	< 0.001	4.8	< 0.001	10.7	0.0018
	FOLFOX + BV	290	22		7.2		12.5	
44	FOLFOX/bFOL/XELOX	147	22-43	NR	6.1-8.7	NR	18.2	NR
	FOLFOX/bFOL/XELOX + BV	213	41-53		8.3-10.3		24.4	
45	FOLFOX/XELOX	701	49	0.99	8.5	< 0.001	-	-
	FOLFOX/XELOX + BV	699	47		11		-	

CRC: Colorectal carcinoma; 5-FU/LV: 5-fluorouracil/leucovorin; IFL: Irinotecan/5-FU/leucovorin; FOLFOX-4: Oxaliplatin/5-FU/leucovorin; BV: Bevacizumab; Pts: Patients enrolled; REF: reference; RR: Response rate; PFS: Progression-free survival; TTP: Time to tumor progression; OS: Overall survival; NR: Not reported.

higher RR [control arm, 17%, (95% CI, 7% to 34%); low-dose arm, 40%, (95% CI, 24% to 58%); high-dose arm, 24%, (95% CI, 12% to 43%)]. Although median survival was 7.7 and 2.3 mo higher in the 5-mg/kg arm and 10-mg/kg arm, respectively, it was not statistically significant. These findings contrast with the effective higher dose administered in other tumors like non-small cell lung cancer (15 mg/kg every three weeks)[33], breast cancer (10 mg/kg every two weeks)[34] and renal cancer (10 mg/kg every two weeks)[35]. Nevertheless, the majority of subsequent CRC studies administered a BV dose of 5 mg/kg. Potential safety concerns observed in this phase Ⅱ study were thrombosis, hypertension, proteinuria, and epistaxis.

In 2004, a large (813 patients) phase Ⅲ, double-blind, randomized trial in patients with untreated metastatic CRC demonstrated that the addition of BV to IFL (irinotecan/5-FU/LV) chemotherapy prolonged OS by 4.7 mo compared with IFL alone (20.3 *vs* 15.6 mo; HR = 0.66, $P < 0.001$)[28]. The one-year survival rate was 74.3% in the group given IFL plus BV and 63.4% in the group given IFL plus placebo ($P < 0.001$). All secondary efficacy end points were also improved with the addition of BV to the chemotherapeutic regimen: PFS increased from 6.2 to

Table 3 Trials for Vatalanib with chemotherapy in metastatic CRC

REF	Regimen	Pts	RR (%)	*P*	PFS or TTP (mo)	*P*	OS (mo)	*P*
58	FOLFOX-4	583	46		7.6			
	FOLFOX-4 + Vatalanib	585	42	NS	7.7	0.118	NR	-
59-60	FOLFOX-4	429	18	NR	4.1	0.026	11.8	0.511
	FOLFOX-4 + Vatalanib	426	19		5.5		12.1	

CRC: Colorectal carcinoma; REF: Reference; Pts: Patients enrolled; FOLFOX-4: Oxaliplatin/5-FU/leucovorin; RR: Response rate; PFS: Progression-free survival; TTP: Time to tumor progression; OS: Overall survival; NS: Statistically nonsignificant; NR: Not reported.

10.6 mo (hazard ratio HR = 0.54; $P < 0.001$), RR increased from 34.8% to 44.8% ($P = 0.004$), and median duration of the response increased from 7.1 to 10.4 mo (HR = 0.62; $P = 0.001$). Grade 3 hypertension was more common during treatment with IFL plus BV than with IFL plus placebo (11.0 percent *vs* 2.3 percent, $P < 0.01$) but it was easily managed with medical treatment. Although the overall incidence of grade 3 or 4 adverse events was higher among patients receiving the combined treatment, the study did not identify hemorrhage, thromboembolism, and proteinuria as possible BV-associated adverse events. Uncommon but serious side-effects of BV included the appearance of gastrointestinal perforations (1.5%), in some instances with fatal outcome[28].

Toxicity derived from antiangiogenic therapy is a main concern in the management of CRC. BV is associated with a two-fold increase in the risk of arterial thromboembolic events, from 2.5% to 5% ($P < 0.01$)[36]. These events consist primarily of acute coronary syndrome, transient ischemic attack and stroke. Patients at risk for these events are those with a prior history of arterial thromboembolism and age older than 65 years. Moreover, BV administration can result in the development of wound dehiscence. However, the risk of wound healing is not increased if the administration of BV with or without chemotherapy is delayed until 28-60 d after primary care surgery[37].

Although the addition of BV to 5-FU-based combination chemotherapy resulted in statistically significant and clinically meaningful improvement in RR, PFS and OS among patients with metastatic CRC, previous studies have suggested that the benefit observed with irinotecan-based schedules might be limited to patients with a performance status (PS) of 0[38]; and certain subgroups, including those with advanced age, impaired PS, low serum albumin, and prior pelvic radiotherapy, may experience significant toxicities when adding irinotecan to 5-5-FU/LV regimens[39]. In this particular population, the combination of BV and 5-FU/LV would remain a potentially useful therapeutic alternative. Two studies led by Kabbinavar *et al*[40,41] addressed this question enrolling patients who were not candidates for irinotecan because of advanced age or poor PS. The results suggested that 5-FU/LV (Roswell Park Schedule[42]) plus BV seems as effective as IFL and might have a better safety profile. Based on all of the previous data, BV became the first anti-VEGF agent to be approved by the FDA for cancer patients.

On June 2006, the FDA granted approval for a labelling extension for BV in combination with intravenous 5-FU-based chemotherapy for the second-line treatment of metastatic CRC. This decision was based on the preliminary results of the E3200 phase Ⅲ trial of the Eastern Cooperative Oncology Group (ECOG). The aim of this randomized, three-arm, multicenter study was to determine the efficacy of infusional 5-FU/LV/oxaliplatin (FOLFOX) with or without BV (10 mg/kg every two weeks) in 829 patients with irinotecan-refractory advanced CRC not previously treated with BV[43]. The median age was 61 years, 49% had an ECOG performance status of 0, and 80% received prior adjuvant chemotherapy. The combination therapy showed an improvement in the OS by 2.1 mo (12.5 *vs* 10.7 mo; $P = 0.0024$) without a significant difference in the toxicity profile. The BV-alone arm was closed at the interim analysis due to a low RR and an apparent lack of activity in this setting. Final analyses of this trial are forthcoming.

Whether the combination of BV with oxaliplatin/5-FU/LV-based chemotherapy regimens will be the best option for first-line therapy for CRC is under investigation in the TREE study[44] and NO16966[45]. The TREE study was previously designed to assess the safety, tolerability and efficacy of each of three oxaliplatin plus fluoropyrimidine regimens without (TREE1 cohort) or with (TREE2 cohort) BV. In the TREE-2 cohort, BV was added to each regimen. With a follow-up of 27 mo, median OS with infusional 5-FU/LV and oxaliplatin (mFOLFOX-6) plus BV was 26.0 mo, 20.7 mo with bolus 5-FU/LV and oxaliplatin (bFOL) plus BV, and 27.0 mo with capecitabine and oxaliplatin (CapeOX) plus BV. Median OS with oxaliplatin-containing regimens without BV in sequential historical cohorts (TREE-1 study), reached 18.2 mo[44]. However, the first large, randomized, multicenter phase Ⅲ trial to evaluate the efficacy of BV in combination with the standard chemotherapy regimen FOLFOX and the XELOX regimen in the first-line treatment of metastatic CRC is the NO16966[45]. Interestingly, in the general treated population, the addition of BV to FOLFOX did not significantly improve PFS (HR = 0.89, $P = 0.1871$). However, 50% of patients discontinued treatment for reasons unrelated to progression of disease. Further analyses focusing on the on-treatment subgroup population revealed that median PFS for XELOX-BV and FOLFOX-BV was 10.4 mo compared to 8.1 mo for XELOX-Placebo and FOLFOX-Placebo (HR = 0.63, $P < 0.0001$). These results demonstrated that the addition of BV to oxaliplatin-based chemotherapy regimens significantly improves PFS. In addition, continuation of BV until disease progression could be necessary to

optimize the contribution of BV to PFS[45].

The activity shown by BV in the metastatic setting justified the evaluation of this antibody in the adjuvant scenario. In the first trial, the National Surgical Adjuvant Breast and Bowel Project C-08 phase Ⅲ trial[46], 2632 patients with stage Ⅱ or Ⅲ colorectal cancer have been randomized to receive mFOLFOX-6 for 12 cycles with or without BV. Patients assigned to BV plus chemotherapy also received an additional 6 mo of BV alone. This trial has already completed accrual. In a second trial recently finished, the AVANT phase Ⅲ study[47], patients with stage Ⅱ or Ⅲ colorectal cancer were randomized to three combination chemotherapy regimens (FOLFOX-4 *vs* FOLFOX-4 plus BV *vs* capecitabine/oxaliplatin plus BV). In addition, a phase Ⅱ clinical trial, the Eastern Cooperative Oncology Group (ECOG) E5202[48], is evaluating the addition of BV in combination with FOLFOX on patients with stage Ⅱ colon cancer at high-risk for recurrence. In conclusion, at this point in time, no evidence supports the actual use of BV in the adjuvant setting in order to prolong survival. The results of these important clinical trials are eagerly awaited.

VATALINIB IN CRC

A second antiangiogenic approach is to target both cancer cells and endothelial cells with small molecules. Similar to BV, VEGFR multitargeted TK inhibitors have been evaluated in combination with chemotherapy in phase Ⅲ trials. The first agent, semaxinib (SU5416, Pharmacia, San Francisco, California) which targets VEGFR-1, VEGFR-2 VEGFR-3, and PDGFR-β did not show any survival benefit when added intravenously to standard chemotherapy in metastatic CRC. In addition worse toxicity in the semaxinib arm was observed[49]. Finally, in a phase I trial that evaluated the combination of semaxinib with cisplatin/gemcitabine in solid tumors, an unexpected high incidence of thromboembolic events was observed which discouraged overall further investigation of this agent[50].

Another novel synthetic agent, with orally bioavailability, vatalanib (PTK787/ZK222584, Novartis, Basel, Switzerland) belongs to the chemical class of aminophthalazines[51]. It is a potent inhibitor of all known VEGFR tyrosine kinases (TK) with greater potency against VEGFR-1 and VEGFR-2[52,53] (Figure 1). It also inhibits other kinases, such as platelet-derived growth factor receptor beta (PDGFR-β) and c-Kit tyrosine kinase. In preclinical studies, vatalanib has shown antitumor activity in subcutaneously implanted human tumor xenografts in nude mice[53]. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and pharmacokinetic (PK) data indicated that vatalanib ⩾ 1000 mg total daily dose is the biologically active dose[54] with a terminal half-life of about 6 h. In view of the short half-life of the drug, a phase I study with vatalanib given twice daily was conducted to exploit the theoretical advantage of maintaining constant drug levels[55]. PK data from this study showed that at equivalent daily doses, drug exposure is comparable with the previous once-daily-dosing schedule[54]; however, the trough levels are significantly higher with the bid dosing. Whether this will translate into improved efficacy is unknown at this time.

Vatalanib has been evaluated in two phase Ⅰ / Ⅱ studies as a single daily-dose in combination with FOLFOX or FOLFIRI, as first-line treatment for patients with metastatic CRC[56,57]. In both studies, vatalanib was safe and well tolerated at doses of 1250 mg/d. Ataxia, expressive dysphasia and dizziness were seen at higher doses when administered in combination with FOLFOX and these were considered dose-limiting toxicities. The combination of vatalanib with chemotherapy significantly affected the PK parameters of SN38, the active metabolite of irinotecan. Indeed, the concentration-time curve (AUC) of SN38 was decreased when vatalanib was added to the FOLFIRI regimen. The relevance of this finding needs further investigation.

Two phase Ⅲ studies have evaluated the administration of vatalanib (single daily-dose of 1250 mg/d) in combination with chemotherapy in CRC (Table 3). A first randomized phase Ⅲ trial (CONFIRM-1) compared the efficacy of vatalanib in combination with FOLFOX versus FOLFOX alone in 1168 patients for first-line treatment of metastatic CRC[58]. The results of the primary endpoint of this trial, PFS, showed a modest benefit of adding vatalanib to FOLFOX without achieving statistical significance (HR = 0.88; P = 0.118). OS has not been reported. The adverse events attributable to vatalanib (hypertension, deep-vein thrombosis, diarrhea and dizziness) were generally reversible and similar to other VEGF pathway inhibitors. No increase in bleeding or bowel perforation compared to placebo was observed. The second phase Ⅲ trial (CONFIRM-2) evaluated the efficacy of vatalanib in combination with FOLFOX *versus* FOLFOX alone in 855 patients with irinotecan-refractory advanced CRC[59,60]. PFS was 1.4 mo significantly longer in the vatalanib arm (5.5 mo *vs* 4.1 mo, HR = 0.83; P = 0.026). No improvement in OS was demonstrated. In the CONFIRM-2 trial, the most frequent grade 3/4 events associated with vatalanib were again hypertension (21% *vs* 5%), diarrhea (16% *vs* 8%), fatigue (14.5% *vs* 6.9%), nausea (11% *vs* 5%), vomiting (9% *vs* 5%) and dizziness (9% *vs* 1%). Two hypotheses have been tried to explain why survival was not affected when adding vatalanib in first and second-line therapy. The first one deals with the short half-life of vatalanib. The once-daily administration of the drug might not be the optimal schedule to maintain constant blood levels of vatalanib, although another study refutes this hypothesis[54]. A second one would be the "off-target" effects, such as targeting PDGFR-β. The inhibition of PDGFR-β could interfere with vascular normalization by blocking perivascular cell recruitment and thus impeding the delivery of chemotherapeutics to chemoresponsive tumors[61].

Major *et al*[62] reported a metanalysis by pooling preplanned strata in CONFIRM-1 (C1) and CONFIRM-2 (C2) trials and showed that patients with high LDH (> 1.5 X ULN) experienced the greatest improvement in PFS for C1 (HR = 0.67; P = 0.01) and for C2 (HR = 0.63; P < 0.001). This finding brings forward an eventual role of LDH in angiogenesis-dependent tumor growth and progression in CRC. Previously, the expression of LDH-5, a LDH isoform, has been linked with distant metastases in

CRC and with the expression of hypoxia inducible factor (HIF)[63]. Furthermore, evidence of a biologic link between tumor LDH, hypoxia and activated VEGF pathway has been described in CRC[64]. LDH, being regulated by the same pathway as VEGF, is expected to reflect a subset of tumors with a high likelihood to bear an activated VEGF signalling pathway. Nevertheless, whether LDH can be used as a surrogate marker for screening patients for TK inhibitor therapy remains an open question. Thus, validation of biomarkers of efficacy of anti-VEGF therapy with the aim of identifying responsive patients and predict the optimal biological dose are imperative.

TARGETED THERAPY COMBINATIONS

Growth factors and their receptors play a pivotal role in the regulation of cancer progression and neovascularization[65], stimulating downstream signaling cascades involved in cell proliferation, survival and antiapoptosis. The expression or activation of epidermal growth factor receptor (EGFR) and ErbB2 are altered in many epithelial tumors, and clinical studies indicate that they have an important role in tumor progression[66]. Inhibiting signaling pathways through EGFR and ErbB2 has become a cornerstone in the treatment of a subgroup of patients with non-small cell lung cancer and breast cancer, respectively. In CRC, cetuximab (IMC C225, Erbitux, ImClone, New York, NY), a monoclonal antibody targeting EGFR[67], has been shown to induce apoptosis of CRC cells[68], and cetuximab in combination with irinotecan (in irinotecan-refractory and EGFR expressing metastatic CRC) was found to reverse resistance to irinotecan, producing a 22.9% RR (BOND-1 Trial)[69,70]. These findings have led to the approval of cetuximab for irinotecan-refractory advanced CRC in the Unites States and, more recently, in Europe.

As it is known, the expression of proangiogenic molecules by tumor cells can be stimulated by EGFR receptor signaling[71]. Furthermore, several studies have shown that EGFR inhibitors reduce VEGF and microvessel density in tumors that regress upon EGFR blockade[72,73]. These results provide a strong rationale for combinations of anti-EGFR agents with angiogenesis inhibitors in CRC.

The safety and efficacy of concurrent administration of BV and cetuximab has been evaluated in a randomized phase Ⅱ trial in patients with irinotecan-refractory metastatic CRC (BOND-2 trial)[74]. Seventy-five patients were assigned to receive either irinotecan/cetuximab/BV (5 mg/kg every other week) or cetuximab/BV. This study presents a similar design to BOND-1 trial with BV included in both arms. The combination of cetuximab/BV, alone or with irinotecan, is tolerable, and RR and median TTP seen with the addition of BV to either arm appear favorable compared to historical controls of the BOND-1 trial. The results of the BOND-2 trial validate the design of the planned intergroup trial CALGB/SWOG 80405[75], which plans to randomize 2289 patients to receive standard chemotherapy with the addition of cetuximab, BV, or both monoclonal antibodies in first-line metastatic CRC. The primary end-point of this trial will be to detect differences in overall median survival.

SMALL-MOLECULE TK INHIBITORS IN CRC

Finally, novel VEGFR and/or PDGFR TK inhibitors with different ranges of nanomolar potencies, selectivities, and pharmacokinetic properties are entering phase Ⅰ/Ⅱ trials for the treatment of cancer[76-78]. In addition, there are now available a series of TK inhibitors that block both the EGFR and the downstream signalling molecules on the one hand and the VEGF receptor TK on the other (Table 1). Zactima (ZD6474, AstraZeneca Pharmaceuticals, Cheshire, UK) is an orally bioavailable, anilinoquinazoline derivative, multitargeted tyrosine kinase inhibitor that targets VEGFR-2, EGFR, and RET tyrosine kinases, and is currently in phase Ⅰ/Ⅱ evaluation for the treatment of cancer[79,80]. Another broad spectrum multitargeted agent, AEE788 (Novartis, Basel, Switzerland), is an oral small-molecule inhibitor of both EGFR and VEGFR tyrosine kinases[81,82]. In preclinical studies, this agent has shown growth and metastases inhibition of human colon carcinoma in an orthotopic nude mouse model[83]. Sorafenib (BAY 43-9006; Nexavar®, Bayer Aktiengesellschaft, Leverkusen-Bayerwerk, Germany, and Onyx Pharmaceuticals Inc., Emeryville, CA) targets VEGFR2 and VEGFR3, PDGFR-β, c-Kit and FLT3 (fms-related tyrosine kinase 3) and the downstream signalling molecule of EGFR known as Raf[84]. This agent efficiently inhibits both tumor-cell proliferation and angiogenesis in preclinical models, and monotherapy treatment has shown efficacy in a phase Ⅲ trial in patients with cytokine-refractory advanced renal carcinoma, which led in 2005 to the approval by the FDA for this indication[85]. In contrast with BV, the monotherapy efficacy demonstrated by Sorafenib could mimic the synergistic effect of the combination of an anti-VEGF antibody and chemotherapy[86]. The activity of Sorafenib and similar agents in the treatment of CRC needs further development. In addition, whether it will be better to target the EGFR and VEGF receptor with two compounds, each targeting one system, or to use these new class of oral duals or broad-spectrum inhibitors, is not known at this time[87].

SUMMARY AND CONCLUDING REMARKS

The increased knowledge of the VEGF signaling network and its implication in the development and progression of CRC, together with the initial positive clinical results observed with anti-VEGF therapies, makes angiogenic targeting an appropriate cancer treatment strategy. Based on the results of the completed phase Ⅲ trials, BV can increase survival when combined with standard chemotherapy in first and second-line therapy of advanced CRC. These findings have led to the approval of BV for the treatment of metastatic CRC. Simultaneously, the activity of BV in combination with 5-FU/LV-based chemotherapy regimens is being evaluated in early disease, a period when angiogenesis might be particularly critical. Results of these trials are eagerly awaited. The initial positive results of anti-VEGF therapy are not accomplished without added toxicity. Side effects of anti-VEGF agents are usually moderate compared with other

therapies, but the etiology is poorly understood. Major safety concerns have been raised by increased morbidity, and a number of treatment-related deaths from bowel perforations and cardiovascular events. Modest elevations in blood pressure occur occasionally and are easily managed with standard antihypertensive medications.

Since multiple growth-controlling pathways may be altered in cancer cells, combination antibody strategies are being explored in advanced CRC. BV is being assessed in combination with cetuximab in irinotecan-refractory metastatic CRC, based on the positive results of anti-EGFR therapies in this context. Preliminary data for this combination shows remarkable results without substantial differences about toxicity. New clinical trials with both targeted strategies in first-line metastatic CRC are recruiting patients. Combination of BV with novel VEGFR and broad-spectrum TK inhibitors also needs to be assessed in the treatment of CRC. One of these VEGFR TK inhibitors, vatalanib, combined with standard chemotherapy has been shown not to improve survival in first and second-line treatment of advanced CRC in both phase Ⅲ trials. New broad-spectrum TK inhibitors, such as Sorafenib, oppositely to the VEGF antibody, have shown promising monotherapy activity in other tumors. The basis of these findings is being extensively evaluated, and the identification of biomarkers to predict therapeutic response and optimal doses of anti-VEGF therapy is urgently needed in order to identify patients who will benefit from antiangiogenic therapy.

Angiogenesis research moves in two directions. In one hand, ongoing and new, well-designed trials will define the optimal clinical application of the actual antiangiogenic agents, and, on the other, intensive efforts in basic research will identify new agents with different antiangiogenic approaches for the treatment of CRC.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Combining chemotherapy and targeted therapies in metastatic colorectal cancer

J Rodriguez, R Zarate, E Bandres, A Viudez, A Chopitea, J García-Foncillas, I Gil-Bazo

J Rodriguez, R Zarate, E Bandres, A Viudez, A Chopitea, J García-Foncillas, I Gil-Bazo, Unit for the Research and Treatment of Gastrointestinal Malignancies, Department of Oncology, Clinica Universitaria, Center for Applied Medical Research, University of Navarra, Spain
Correspondence to: Javier Rodriguez, MD, Unit for the Research and Treatment of Gastrointestinal Malignancies, Department of Oncology, Clinica Universitaria, Center for Applied Medical Research, University of Navarra, Spain. jrodriguez@unav.es
Telephone: +34-948-255400 Fax: +34-948-296500
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Abstract

Colorectal cancer remains one of the major causes of cancer death worldwide. During the past years, the development of new effective treatment options has led to a considerable improvement in the outcome of this disease. The advent of agents such as capecitabine, irinotecan, oxaliplatin, cetuximab and bevacizumab has translated into median survival times in the range of 2 years. Intense efforts have focused on identifying novel agents targeting specific growth factor receptors, critical signal transduction pathways or mediators of angiogenesis. In addition, several clinical trials have suggested that some of these molecularly targeted drugs can be safely and effectively used in combination with conventional chemotherapy. In this article we review various treatment options combining cytotoxic and targeted therapies currently available for patients with metastatic colorectal cancer.

Key words: Targeted therapy; Chemotherapy; Combinations; Clinical trials; Colorectal cancer

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INTRODUCTION

Chemotherapy remains the cornerstone of treatment of metastatic colorectal cancer (mCRC) and, with the exception of a minority of patients (pts) who are candidates for salvage surgery, the goal of chemotherapy is palliation. Remarkable and clinically relevant advances have been made in the last 5 years in the treatment of this disease, essentially owing to the introduction of combination chemotherapy regimens containing oxaliplatin and irinotecan (CPT-11)[1]. The addition of either drug to 5-fluorouracil/leucovorin (5-FU/LV) proved to significantly increase overall response rates and survival times. Indeed, median overall survival is highly correlated with the percentage of patients who receive the three cytotoxic agents in the course of their disease. Results from a Phase Ⅲ study by Falcone *et al*[2] suggested that the up-front use of a triplet combination of irinotecan, oxaliplatin and 5-FU/LV significantly improved the outcome in terms of response rate (RR) and survival times compared to a standard doublet of irinotecan and 5-FU/LV.

Interestingly, with the more recent incorporation of bevacizumab and cetuximab into the treatment armamentarium, the median overall survival (OS) has doubled from 12 mo to approximately 2 years in Phase Ⅲ trials. In fact, most recent trials that attempt to expose patients to all five drug classes (fluoropyrimidines, irinotecan, oxaliplatin, bevacizumab and anti-EGFR antibody) target an OS well over 2 years. In this review we will summarize some of the available therapeutic repertoire based on targeted therapies in combination with chemotherapy for patients with mCRC.

COMBINING CHEMOTHERAPY AND EGFR-TARGETED THERAPIES

The epidermal growth factor receptor (EGFR), a transmembrane tyrosine kinase, is one of four members of the HER receptor family. This receptor is overexpressed in a number of solid tumors of ectodermal origin, including colon adenocarcinoma[3]. EGFR over expression has been correlated with disease progression, poor prognosis and reduced sensitivity to chemotherapy[4]. Therefore, several strategies have been developed to target EGFR, including small molecule tyrosine kinase inhibitors and monoclonal antibodies[5].

Cetuximab-based combination therapy

Cetuximab is the most advanced monoclonal antibody against EGFR in clinical development. Since preclinical

and early clinical studies suggested that Cetuximab might revert irinotecan resistance in CRC both *in vitro* and *in vivo*, a phase Ⅱ trial of cetuximab with irinotecan was performed in patients with EGFR positive colorectal cancer that was refractory to both 5-fluorouracil (5-FU) and Irinotecan. Among the 120 patients treated with this regimen, overall response rate was 22.5%[6].

To confirm these clinical findings, 329 EGFR-positive, irinotecan-refractory mCRC patients were randomized in a 2:1 ratio to receive cetuximab plus irinotecan (arm A; $n = 218$) or cetuximab alone (arm B; $n = 111$) with the option to switch to the combination of cetuximab with irinotecan after failure of cetuximab as a single agent. Both the response rate (22.9% *vs* 10.8%, $P = 0.007$) and the median time to progression (4.1 *vs* 1.5, $P < 0.001$) favored the combination arm. Although no survival benefit was observed for arm A, cetuxibab was demonstrated to have clinically significant activity when given alone or in combination with irinotecan and consequently received FDA approval[7].

More recently, MABEL trial[8] investigated the combination of cetuximab and CPT-11 at a dose and schedule as pre-study in an uncontrolled, multicenter study including 1123 mCRC pts with detectable EGFR. 64% of the patients had received ⩾ 2 lines of chemotherapy. 76% had also been pretreated with cetuximab. The estimated median survival was 9.2 mo at as expense of an acceptable toxicity profile, including grade 3-4 diarrhea (20%) acne-like rash (19%), neutropenia (9%) and asthenia (8%). MABEL clearly confirmed in a wider setting the efficacy and safety or C225 plus CPT-11 seen in previous studies. Similarly, EPIC trial is a randomized phase Ⅲ trial comparing cetuximab plus irinotecan to irinotecan as second line therapy in patients with EGFR-expressing mCRC who have failed first line oxaliplatin in combination with a fluorpyrimidine. Accrual is currently ongoing[9].

Cetuximab-based combinations as salvage therapy: Several trials have addressed the potential of cetuximab-based combinations in heavily pretreated patients. Vincenzi *et al*[10] evaluated the efficacy of cetuximab plus oxaliplatin in patients previously failed on an oxaliplatin-based regimen in first line, irinotecan-based regimen in second line, and cetuximab plus irinotecan in third line. No objective clinical response was identified after the interim analysis planned according to the two-staged Simon accrual design. The same group[11] evaluated the activity of cetuximab and weekly irinotecan (90 mg/m^2) in patients refractory to one oxaliplatin-based chemotherapy regimen (Capecitabine + Oxaliplatin or FOLFOX Ⅳ regimen, as first line) and one Irinotecan-based based-chemotherapy (FOLFIRI regimen, as second-line chemotherapy) for at least 2 mo. Overall response rate was 25.4% (95% CI: 21.7%-39.6%); 38.2% (95 CI: 18.6%-39.8%) of patients showed a disease stability as the best response. The median time to progression was 4.7 mo (95% CI: 2.5-7.1 mo) and the median survival time was 9.8 mo (95% CI: 3.9-10.1 mo). The most common G3-4 noncutaneous side toxicities were diarrhoea (16.4%), fatigue (12.7%), stomatitis (7.3%) and skin toxicity (32.6%). A statistically significant ($P = 0.006$) association between the cutaneous toxicity and both tumour response and time to progression was observed. The authors also identified a borderline significant difference in terms of overall survival.

The combination of Cetuximab plus FOLFIRI has been prospectively evaluated in 41 EGFR expressing mCRC pts refractory to prior FOLFIRI for metastatic disease[12]. Most of the patients were treated in third line. A 20% overall response rate was recorded, with a median PFS of 4.3 mo and a median overall survival of 5 mo.

Cetuximab-based combinations in front-line therapy: Cetuximab established activity in the salvage setting prompted its incorporation to first-line combination therapy. Available preliminary data from Phase Ⅱ trials combining cetuximab with either irinotecan or oxaliplatin-based chemotherapy have shown very encouraging activity. CALGB 80203[13] randomized untreated mCRC patients to FOLFOX or FOLFIRI with or without C225 independent of EGFR status. ORR was similar in the FOLFIRI or FOLFOX arms, while C225 containing arms had a higher ORR (49% *vs* 33%, $P = 0.014$) when compared to non cetuximab containing arms. No significant differences in grade 3 diarrhea or any grade 4 toxicity were seen with the addition of C225. Preliminary results of the combination of C225, capecitabine (800 g/m^2 bid po on d 1 to 14) and Irinotecan (200 g/m^2 i.v on d 1) *vs* C225 combined with capecitabine (1000 mg/m^2 bid in d 1-14) and oxaliplatin (130 g/m^2 on d 1) reported an overall response rate of 41% (95%; 22% to 61%) and 71% (95%; 48% to 89%) respectively, with both arms showing a manageable toxicity profile[14].

Promising results have also been reported[15] combining cetuximab with (AIO) infusional 5-FU/FA plus irinotecan regimen in EGFR-expressing mCRC.Grade 3 or 4 toxicities were acne-like rash (38%), diarrhea (29%), cardiovascular events (20%) and nausea/vomiting (5%). Objective responses were observed in 67% of the patients. The median time to progression was 9.9 mo and the median survival time was 33 mo.

The combination of cetuximab with modified FOLFOX 6 in 83 chemo-naive mCRC pts with positive or undetectable EGFR expression show a preliminary ORR of 53%[16]. Main grade 3-4 toxicities included neutropenia (38%), diarrhea (10%), rash (10%) and neurotoxicity (7%). The combination of FOLFOX-4 plus C225[17] has also been evaluated in 47 EGFR-expressing mCRC, with a reported ORR of 68%. Grade 3-4 adverse events included acne-like rash (18%) diarrhea (7%), nausea and vomiting (4%) and anemia (4%).

Preliminary results of the OPUS trial[18], a randomized phase Ⅱ study in the first line treatment of mCRC, confirmed the superiority of FOLFOX plus cetuximab *vs* FOLFOX in terms of overall response rate (45.6% *vs* 36.8%).

These small trials supported the conduct of a multicenter Phase Ⅲ clinical trial that compared FOLFIRI plus Cetuximab with FOLFIRI alone in 1217 EGFR-expressing chemotherapy-naive patients. Cetuximab plus FOLFIRI significantly increased response rate and progression-free survival, reducing the relative risk of progression by approximately 15%[19].

Panitumumab-based combination therapy

Panitumumab is a fully human IgG2 monoclonal antibody directed against the epidermal growth factor receptor. Its use in combination with IFL and FOLFIRI in first line treatment of metastatic CRC has been evaluated in a multicenter, single arm, phase 2 trial[20]. Panitumumab was given at a weekly dose of 2.5 mg/kg i.v. over 60-90 min followed by chemotherapy. The combination with IFL was considered too toxic, with grade 3-4 diarrhea in 47% of the patients. The FOLFIRI plus panitumumab combination was associated with a more manageable side effect profile with grade 3-4 diarrhea in 25% of the pts and grade 3-4 hypomagnesemia in 8%. Skin and nail toxicities occurred in at least 20% of patients but were rarely severe (grade 3 in 2 out of 24 pts). The objective response rate with FOLFIRI plus panitumumab was 66%, with a disease control rate of 79%. Median progression free survival was 10.9 mo. Further investigation of FOLFIRI with an every two weeks schedule of panitumumab is ongoing in randomized phase 3 trials.

Cetuximab-induced papulopustular skin rash is thought to be mechanism- and dose-related, and may be a surrogate indicator of an adequate degree of receptor saturation by cetuximab. The possibility of increasing Cetuximab efficacy by inducing skin rash has been recently confirmed. Cetuximab dose escalation up to 500 mg/m^2 improves response rate in patients with absent or slight skin reaction on standard dose treatment[21].

Future directions

Large studies validating molecular predictive markers are needed in order to identify the subset of patients more likely to respond to EGFR-targeted therapies. Candidate markers include total and phosphorylated EGFR, total and phosphorylated forms of AKT, mitogen-activated protein kinase (MAPK), mitogen-activated protein/ERK (MEK), ERK, signal transducers and activators of transcription (STAT), PTEN and mTOR[22] Although EGFR gene copy number has also been proposed[23], EGFR amplification, measured by FISH is a rare event (4%) in colorectal cancer[24]. Other potential predictive markers are k-ras[25] cyclin D1 A870G polymorphisms[26], HER-2 expression[27] or higher gene expression levels of VEGF[28]. More recently, a combination of various predictive biomarkers has retrospectively been able to identify subsets of patients more likely to benefit from cetuximab therapy[29]. In addition, several polymorphisms in genes involved in the EGFR and angiogenesis pathway have been associated with clinical outcome[30]. Prospective studies are clearly needed to confirm these preliminary findings.

EGFR tyrosine kinase inhibitor (TKI)-based combination therapy

Gefitinib: Gefitinib (ZD1839) selectively inhibits the EGFR tyrosine kinase and has approximately 100-fold greater potency against EGFR compared with other tyrosine or serine/threonine kinases. Unlike cetuximab, gefitinib does not induce EGFR internalization or degradation in CRC cells, nor does it reduce EGF binding sites or EGFR protein content. Both *in vitro* and *in vivo* studies indicated that gefitinib monotherapy had antitumor activity in some CRC cell lines[31]. However, phase I / Ⅱ clinical studies in patients with mCRC indicated that gefitinib had negligible activity[32,33]. Preclinical suggestions of a supra-additive, growth-inhibitory effect of gefitinib and a wide variety of cytotoxic drugs with different mechanism(s) of action[34] prompted several trials of gefitinib in combination with chemotherapy in mCRC patients.

Gefitinib plus fluoropyrimidines: In preclinical models a strong synergistic interaction between gefitinib and 5'-deoxy-fluorouridine (5'-DFUR) was demonstrated when ZD1839 was applied before or concurrently with 5'-DFUR[35]. Subsequently, the combination of intermittent gefitinib (250-500 mg/d on d 1-14) plus 5-FU/LV administered as a bolus in a dose-reduced Mayo Clinic regimen (370/20 mg/m^2) on d 8-12 with 5-FU and leucovorin as first-line therapy in mCRC was tested, with no evidence of cumulative toxicity or major drug-drug pharmacokinetic interactions[36]. In the second part of the study, gefitinib was administered continuously at 500 mg/d, and 5-FU/LV was added to the schedule on d 8-12 and 36-40. Overall response rate was 23%, with the most common toxicities being rash and diarrhea.

Preliminary results from a small phase I / Ⅱ trial combining gefitinib 250-mg daily with capecitabine 1000-1250 mg twice daily after failure of first-line therapy[37] also suggest some evidence of activity .

Gefitinib plus irinotecan-based therapy: A dose-finding trial of irinotecan plus gefitinib in mCRC patients pretreated with fluoropyrimidine-based chemotherapy defined irinotecan given at a dose of 225 mg/m^2 every 3 wk plus gefitinib at a dose of 250 mg/d as the maximun tolerated dose (MTD) of this regimen[38]. Dose-limiting toxicities (DLTs), such as neutropenia and diarrhea, occurred at unexpectedly low doses of irinotecan. Disease stabilization was achieved in 21% of the patients.

The combination of gefitinib plus FOLFIRI in both chemotherapy-naive mCRC patients[39] and as salvage therapy[40] was considered too toxic despite reduced weekly doses of 5-FU, LV, and irinotecan.

Gefitinib plus oxaliplatin-based therapy: Gefitinib plus FOLFOX has been tested in both the first line and the salvage setting. Kuo *et al*[41] reported data on a phase Ⅱ study of one cycle of FOLFOX-4, and then additional cycles of FOLFOX-4 with 500 mg/d of gefitinib in 27 patients with documented progressive colorectal cancer after at least one chemotherapeutic regimen (usually irinotecan based). 33% of the patients achieved objective responses, whereas 48% had stable disease for a prolonged period. Response rates did not differ depending on number of prior regimens. Median event-free survival was 5.4 mo, and overall survival was 12 mo. Another feasibility study assessed the combination of gefitinib (250 mg/d) plus capecitabine (2000 mg/m^2 per day, d 1-15) plus oxaliplatin (120 mg/m^2 every 3 wk for six courses) as first-line treatment in patients with mCRC[42]. The most common grade 3 adverse events were diarrhea and neutropenia. A clinical benefit rate of 58% has been noted.

Overall, toxicity rates with the addition of gefitinib to an oxaliplatin-fluoropyrimidine combination are markedly more favorable than with the irinotecan-based regimens, although higher incidences of grade Ⅲ or Ⅳ diarrhea, nausea, and vomiting than with FOLFOX alone are noted. Further studies of TKI-based therapy for CRC are planned or recruiting.

Erlotinib: Erlotinib, an orally reversible TKI reduces intratumoral EGFR autophosphorylation[43] with no effect on EGFR expression or surface receptor density. Evidence of single agent erlotinib activity in mCRC patients derived from disease-specific phase Ⅱ studies[44] led to the design of several trials in combination with chemotherapy.

Tarceva plus fluoropyrimidines: Additive activity of capecitabine and erlotinib in tumor models[45] supported a phase 2 trial evaluating the combination of erlotinib 150 mg daily with capecitabine 1000 mg/m^2 bid. for 14 d every 3 wk in chemotherapy-naive mCRC patients. Grade 3 diarrhea (30%) grade 3 renal insufficiency (10%) and grade 3 hiperbilirrubinemia (10%) were the most troublesome toxicities. Regarding efficacy, no complete responses were achieved whereas disease control rate was 34%[46].

Tarceva plus oxaliplatin: Meyerhardt *et al*[47] reported on the results of a triplet regimen of erlotinib, 100 mg/d, capecitabine, 1650 mg/m^2 per day (d 1-14), and oxaliplatin, 130 mg/m^2 every 3 wk in 32 patients mostly pretreated with an irinotecan-containing regimen. By intent-to-treat analysis, 25% of the patients experienced a partial response and 44% had stable disease for at least 12 wk. 29% of the patients discontinued study therapy due to toxicity.

Other TKIs-based combinations

EKB-569, an irreversible dual inhibitor of the EGFR and HER-2 tyrosine kinases, inhibits the growth of tumor cells that overexpress EGFR or HER-2 *in vitro* and *in vivo*[48]. Dose-limiting toxicities with EKB-569 plus FOLFIRI in 47 chemotherapy-naive mCRC patients[49] were grade 3 diarrhea and grade 3 fatigue. The MTD was selected as 25 mg EKB-569. The response rate was 38% and the clinical benefit rate was 85%. EKB-569 treatment resulted in complete inhibition of pEGFR and significant inhibition of pMAPK in both skin samples (11 patients) and tumor samples (three patients) with no change in pAkt activity.

In a dose-escalation study[50] with FOLFOX-4 plus EKB-569, 25-75 mg/d, starting from d 3, DLTs were observed with EKB-569 at a dose of 35 mg/d (grade Ⅲ diarrhea and febrile neutropenia), leaving an MTD of 25 mg/d. The most common grade Ⅲ or Ⅳ adverse events were neutropenia (32%; 9 of 29 patients) and diarrhea (8%; 2 of 29 patients).

COMBINING CHEMOTHERAPY AND VEGF-TARGETED THERAPIES

Bevacizumab

Clinical development of Bevacizumab (BV) has rapidly progressed to Phase Ⅲ trials after a preliminary randomized Phase Ⅱ trial in which 104 previously untreated mCRC patients were randomized to two doses of BV (5 and 10 mg/kg) in addition to bolus 5-FU/LV (high dose, Rosewell-Park regimen) or to 5-FU/LV alone[51]. The combination of 5-FU/LV with low-dose BV (5 mg/kg every 2 wk) demonstrated superiority compared with the control monotherapy arm and to the BV-containing arm at a higher dose. These results provided the rationale for the key front-line Phase Ⅲ study by Hurwitz *et al*[52] which demonstrated superiority of IFL plus BV over IFL plus placebo in terms of RR (45% *vs* 35%), PFS(10.6 mo *vs* 6.2 mo) and OS(20.3 mo *vs* 15.6 mo). A subanalysis of this trial has recently stablished the benefit of Bevacizumab in mCCR patients with poor conditions[53].

The second trial (E3200) was a second-line Phase Ⅲ study, designed for patients who already failed an irinotecan-containing therapy and did not receive BV in first-line treatment[54]. Initially, the study included three randomization arms: FOLFOX4 plus BV 10 mg/kg, FOLFOX4 alone or BV 10 mg/kg alone. The BV single-agent arm was closed ahead of time since it was clearly inferior to both other arms (RR 3% and PFS 2.7 mo). The results again largely favored the BV-containing arm, especially in terms of RR (21.8% *vs* 9.2%, $P < 0.0001$) and PFS (7.2 mo *vs* 4.8 mo, $P < 0.0001$). The primary end point of the study was reached, since a statistically significant increase in median survival was obtained in the experimental arm (12.5 mo *vs* 10.7 mo, $P < 0.0024$).

Finally, updated results of N016966, a randomized phase Ⅲ trial evaluating the addition of bevacizumab to oxaliplatin-based first line chemotherapy have been reported. Bevacizumab-containing arms demonstrated a significant benefit in terms of progression-free survival, although overall response rate did not significantly differ[55].

More recently, several phase Ⅱ trials have addressed the feasibility and activity of bevacizumab when combined with various cytotoxic regimens. The First BEATrial[56] enrolled 1927 chemotherapy-naïve patients treated with a combination of bevacizumab and several first-line chemotherapies, including FOLFOX, FOLFIRI and XELOX. Median PFS was 10.4 mo. Combinations of XELOX or XELIRI plus bevacizumab have yielded tumor control rates in the range of 80% as front-line therapy for mCRC[57].

In contrast to its efficacy when used in combination with first- and second-line chemotherapy, activity of bevacizumab in chemoresistant disease has been disappointing. Chen *et al*[58] developed a treatment referral center (TRC) protocol (TRC-0301) for patients with mCRC in the third-line setting with the aim of evaluating the safety and activity of BV plus FU/LV in patients progressed after treatment with both irinotecan-based and oxaliplatin-based chemotherapy regimens. Independent review confirmed one PR (1%; 95% CI, 0% to 5.5%). Median PFS in this cohort was 3.5 mo (95% CI, 2.1 mo to 4.7 mo) and median OS was 9.0 mo (95% CI, 7.2 mo to 10.2 mo). The authors conclude that BV, alone or in combination with an ineffective chemotherapy in the third-line setting, is likely to be of minimal, if any, clinical benefit.

An important question that remains unresolved is

whether to continue bevacizumab with second-line therapy following failure of a bevacizumab-containing first-line regimen. Although retrospective data from the BRiTE trial suggest that the use of bevacizumab beyond first progression correlate with an improved survival, more mature data are required to draw any firm conclusion[59].

VEGF Tyrosine kinase inhibitors (TKI)-based combination therapy

Tyrosine kinase inhibitors of vascular endothelial growth factor receptors (VEGFRs) are low molecular weight, ATP-mimetic proteins that bind to the ATP-binding catalytic site of the tyrosine kinase domain of VEGFRs, resulting in a blockade of intracellular signaling. Several of these molecules have entered clinical evaluation.

Semaxanib: Semaxanib is a small, lipophilic, synthetic molecule that inhibits VEGFR-1, and -2 tyrosine kinases[60]. A promising response of 31.6% was observed with semaxanib at two different dose levels, 85 and 145 mg/m^2 twice weekly in combination with fluorouracil plus leucovorin as first-line therapy for 28 patients with mCRC[61]. However, a randomized, multicenter, phase Ⅲ trial failed to show any improvement in clinical outcome with semaxanib in combination with fluorouracil and leucovorin (Roswell Park regimen) *versus* fluorouracil and leucovorin alone as first-line therapy for 737 mCRC patients; moreover, worse toxicity in the semaxanib arm (in terms of diarrhea, cardiovascular events, vomiting, dehydration, and sepsis) was observed[62].

Vatalanib: Valatanib is a synthetic, low molecular weight, orally bio-available agent that inhibits all known VEGFR tyrosine kinases, platelet-derived growth factor receptor beta (PDGFR-β) and c-Kit tyrosine kinase[63].

Vatalanib was evaluated in two phase Ⅰ/Ⅱ studies as a single daily dose in combination with FOLFOX-4 or FOLFIRI. In the first study, the pharmacokinetics and toxicity profiles of both vatalanib and FOLFOX-4 were unaffected by co-administration[64]. The reported response rate was 54%, with a median PFS of 11 mo and an estimated median OS time of 16.6 mo . In the second study[65], co-administration of vatalanib at 1250 mg/d with FOLFIRI had minor effects on irinotecan exposure but lowered by 40% the AUC of SN-38 in patients' serum. The response rate was 41%, with a median PFS duration of 7.1 mo and a median OS time of 24.3 mo. Two large, randomized, double-blinded, placebo-controlled, phase Ⅲ trials compared the efficacy of oral vatalanib in combination with FOLFOX-4 with FOLFOX-4 alone in patients with mCRC, and none of them met the primary end points. In the CONFIRM-2 trial, the addition of PTK/ZK to FOLFOX-4 in previously treated mCRC did not meet the primary end points of the study. OS was 12.1 mo in the PTK/ZK arm and 11.8 mo in the placebo arm. The overall response rate was, respectively, 18.5 and 17.5%. PFS was significantly longer in the PSK/ZK arm (5.5 mo *vs* 3.8, $P = 0.026$) As in confirm 1 trial, patients with pretreatment high LDH showed a strong improvement in PFS[66]. Adverse events were similar to those of the CONFIRM-1 trial. Thrombotic and embolic events of all grades occurred in 6% of the patients treated with PTK/ZK *vs* 1% in the placebo arm. Trying to further analyze the relation between LDH levels and clinical outcome with PTK/ZK, Fixed paraffin embedded tumor samples from 36 mCRC not included in the CONFIRM trials were analyzed and tumor gene expression correlated with serum levels of LDH in the same group of patients. Intratumoral levels of LAMA, hipoxia inducible factor 1 (HIF-1), Glut-1 and VEGFA were significantly correlated. Moreover, patients with high serum LDH showed increased intratumoral gene expression of VEGFA, supporting the hypothesis of serum LDH levels as a surrogate maker for activation of the hypoxia inducible factor related genes in the tumor[66].

AZD2171: Preliminary data of a phase I evaluation of AZD2171, a highly potent and selective inhibitor of VEGFR signaling, in combination with several chemotherapy regimens including FOLFOX-6 and CPT-11, has shown some evidence of activity[67].

Vandetalib: Vandetalib, a once-daily oral inhibitor of VEGFR-dependent tumor angiogenesis, EGFR- and RET-dependent tumor proliferation, in combination with FOLFOX6[68] or FOLFIRI[69] has also shown some evidence of activity in mCRC, with diarrhea and neutropenia being the most frequent grade 3 toxicities.

Future directions

So far, clinical, biochemical, and molecular markers have failed to discriminate which patients are more likely to benefit from bevacizumab-containing regimens. An analysis of predictive markers showed indeed that bevacizumab increased the activity of irinotecan plus FU/LV regardless of the level of VEGF expression, thrombospondin expression, and microvessel density[70]. Mutations of *k-ras*, *b-raf*, and *p53* could not predict for a prolonged survival on bevacizumab plus irinotecan plus bolus FU/LV[71]. Recently, Shaye *et al* evaluated functionally significant polymorphisms of genes involved in the angiogenesis pathway in mCRC patients who receive bevacizumab as part of their front-line therapy. There were statistically significant associations between genomic polymorphisms of KDR, CXCR2, MMP7, leptin and both progression-free survival and response rate. Hopefully, prospectively collected samples from patients enrolled onto cooperative group studies and the development of selective micro arrays to define the angiogenesis-related genes in individual tumors, and at different stages of therapy and tumor progression may allow improved therapeutic efficacy.

COMBINATION OF TARGETED THERAPIES

The assumption that most advanced solid tumors derive their growth advantage from more than a signaling pathway and the significant level of compensatory cross talk among receptors within a signaling network as well as with heterologous receptor systems has provided the basis of a combined molecular targeting approach, in which more than one class of inhibitor is applied simultaneously.

A phase Ⅱ study with the combination of FOLFOX,

bevacizumab (5 mg/kg) and erlotinib (150 mg/d) every two weeks in 31 chemotherapy naive mCRC patients has been recently conducted. Grade 3-4 adverse events included diarrhea (29%) neutropenia (29%) rash (18%), fatigue (14%) and neuropathy (11%) 78% of the patients had at least one grade 3-4 toxicity. Remarkably, as much as 42% of the patients came off for toxicity. Similar results have been reported in the DREAM-OPTIMOX3 study, with a 70% incidence of grade 3-4 toxicity when adding erlotinib to a combination of bevacizumab and XELOX[73].

A phase Ⅱ trial of FOLFOX plus bevacizumab and cetuximab in 67 chemotherapy-naïve mCRC patients yielded a 55% response rate, with a median PFS of 9.6 mo and 71% of the patients progression-free for at least 8 mo[74].

The combination of FOLFOX or FOLFIRI with panitumumab and AMG706, an oral multikinase inhibitor targeting VEGF, PDGF and Kit receptors has been tested in 45 mCRC patients, with no apparent PK/PD interactions and an overall response rate in the range of 50%[75].

Based on these results, combinations of monoclonal antibodies are currently being actively tested in first-line therapy of mCRC. The Cancer and Leukemia Group B (CALGB)/South West Oncology Group (SWOG) Intergroup 80405 Phase Ⅲ trial randomizes patients to either cetuximab or bevacizumab, or both antibodies in combination, with the oncologist's choice of FOLFOX or FOLFIRI. In addition, the Panitumumab Advanced Colorectal Cancer Evaluation (PACCE) trial is currently evaluating the efficacy of FOLFOX or FOLFIRI (depending on the investigator choice) plus BV, versus the same combination plus panitumumab.

OTHER TARGETED THERAPIES-BASED COMBINATIONS

Cell cycle inhibitors

Kortmansky *et al*[76] reported the results of the combination of 5-FU and UCN-01, a selective inhibitor of a number of serine-threonine kinases, including calcium and phospholipid-dependent protein kinase C and cell cycle specific kinases, among 35 patients with advanced solid tumors, the majority of them with a diagnosis of mCRC. No objective responses were observed, although eight patients had stable disease. Most of the patients with stable disease had previously received and progressed on 5-fluorouracil. There was minimal toxicity attributed to the combination, although expected toxicities associated with UCN-01 were observed.

Apoptosis modifiers

Bcl-2 plays a pivotal role in the regulation of caspase activation and apoptosis. Its overexpression is found in 30%-94% of clinocopathological colorectal carcinoma specimens and confers a multidrug resistant phenotype in several cell lines. In support of this data, antisense oligonucleotide therapy directed against bcl-2 was shown to significantly enhance the chemosensitivity in several cancer cell lines compared with controls *in vitro*. A recently published phase I trial assessed the feasibility and pharmacokinetic behaviour of the combination of oblimersen sodium, a phosphorothioate antisense oligonucleotide that hybridizes to the first six codons of the bcl-2 open reading frame mRNA, with CPT-11 in 20 pts with mCRC. Among them, 1 pt experienced a PR while 10 additional patients had stable disease lasting 2.5-10 mo. The authors recommend oblimersen at 7 g/kg/d, d 1-8 with CPT-11 280 mg/m^2 on d 6 once every 3 wk was the RD for further development in phase Ⅱ trials[77].

Proteasome inhibitors

The proteasome inhibitor Bortezomib (PS-341), at a dose of 1.3 mg/m^2 administered twice weekly every 21 d in pretreated patients with mCRC did not prove to have clinical activity[78].

The main nonhematologic toxicities were elevation of alkaline phosphatase, constipation, fatigue, nausea, and sensory neuropathy. A phamacokinetic and phamacodynamic analysis of topotecan plus PS-341 in 22 patients with advanced solid malignancies found that, with the addition of PS-341, peripheral blood mononuclear cells (PBMC) topoisomerase I levels got stabilised or increased. These findings suggest that PS-341 may overcome resistance to topoisomerase I inhibitors, since in vitro exposure to campothecin results in down-regulation of the target enzyme. Preliminary data of the combination of FOLFOX4 plus bortezomib in mCRC patients[79] show evidence of clinical activity, with bortezomib at a dose of 1 mg/m^2 being the RD for phase Ⅱ trials.

COX inhibitors

Numerous clinical trials are ongoing to test the efficacy of nonsteroidal anti-inflammatory COX-2 inhibitors in combination regimens for therapy of advanced solid tumors[80]. Preliminary data on the combination of rofecoxib (50 mg/d) with weekly irinotecan and infusional fluorouracil demonstrated a good tolerability up to the irinotecan dose of 125 mg/m^2/wk. The phase Ⅱ study showed a 36.7% objective response rate, a clinical benefit of 76.7% and a median TTP and overall survival of 4 and 9 mo, respectively. The combination was feasible and safe, with a reduced rate of mucositis and diarrhea[81].

However, in the BICC-C trial[82], addition of celecoxib to several Irinotecan/fluorpyrimidine combinations did not impact safety or efficacy. Results of larger studies seem warranted.

Histone deacetylase inhibitors

Histone acetylation by histone acetyltransferases is important for promoting the action of several transcription factors. Acetylation facilitates binding of transcription factors to specific target DNA sequences by destabilizing nucleosomes bound to the promoter region of the target genes[83].

Vorinostat, a novel histone deacetylase inhibitor that potentiates 5-FU through a decrease in thymidylate synthase (TS) expression has been tested in combination with FOLFOX, in a phase I study that enrolled mCRC patients who had failed prior FOLFOX, irinotecan and

cetuximab therapy. Tolerance was acceptable, and some evidence of both, clinical activity (SD in some patients) and biological activity (down regulation of TS) are suggested[84].

mTOR inhibitors

Rapamycin displays potent antimicrobial and immunosuppressant effects as well as antitumor properties. Rapamycin's antiproliferative actions are due to it's ability to modulate key signal transduction pathways that link mitogenic stimuli to the synthesis of proteins necessary for the cell cycle to progress from the G1 to S phase[85].

Rapamycin clinical development has been hampered due to the poor aqueous solubility and chemical stability of the macrolide. CCI-779, a rapamycin ester derived from 2, 2-bis (hydroxymethyl) propionic acid, is one analog that was selected for further development due to its promising pharmacological, toxicological and antitumor profiles[86].

A phase I study of escalating doses of CCI-779 in combination with 5-FU/leucovorin in patients with advanced solid tumors, including mCRC reported preliminary evidence of activity including 1 complete response in a patient with mCRC receiving the 15 mg/m^2 dose and several patients with stable disease of a maximum duration of 12 mo. Further studies are required to determine appropriate regimens with this combination treatment[87].

CONCLUSION

In conclusion, the biological agents have clearly increased the therapeutic armamentarium of patients with metastatic CRC and offer also prospects for an increased chance of a longer survival. Eventually, the availability of more predictive biological factors may allow oncologists to tailor individualized targeted combination therapy to a specific patient with a specific tumor. However, the cost of novel therapies for mCRC is particularly high. Such a heavy economical burden may be counterbalanced either by a very significant breakthrough in treatment efficacy or by selection of patients with a higher chance of responding to a specific treatment.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Epidermal growth factor receptor inhibitors in colorectal cancer treatment: What's new?

M Ponz-Sarvisé, J Rodríguez, A Viudez, A Chopitea, A Calvo, J García-Foncillas, I Gil-Bazo

M Ponz-Sarvisé, J Rodríguez, A Viudez, A Chopitea, J García-Foncillas, I Gil-Bazo, Oncology Department, Clínica Universitaria, Universidad de Navarra, Pamplona 31008, Spain
A Calvo, J García-Foncillas, I Gil-Bazo, Division of Oncology, CIMA, Spain
Correspondence to: Ignacio Gil-Bazo, MD, PhD, Department of Oncology, University Clinic, University of Navarra, Pio XII, 36, Pamplona 31008, Spain. igbazo@unav.es
Telephone: +34-948-255400 Fax: +34-948-255400
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Abstract

Colorectal cancer constitutes one of the most common malignancies and the second leading cause of death from cancer in the western world representing one million new cases and half a million deaths annually worldwide. The treatment of patients with metastatic colon cancer comprises different regimens of chemotherapeutic compounds (fluoropyrimidines, irinotecan and oxaliplatin) and new targeted therapies. Interestingly, most recent trials that attempt to expose patients to all five-drug classes (fluoropyrimidines, irinotecan, oxaliplatin, bevacizumab and cetuximab) achieve an overall survival well over 2 years. In this review we will focus on the main epidermal growth factor receptor inhibitors demonstrating clinical benefit for colorectal cancer mainly cetuximab, panitumumab, erlotinib and gefitinib. We will also describe briefly the molecular steps that lie beneath them and the different clinical or molecular mechanisms that are reported for resistance and response.

Key words: Epidermal growth factor receptor inhibitors; Cetuximab; Panitumumab; Erlotinib; Gefitinib; Metastatic colorectal cancer; Tyrosine kinase inhibitors; Monoclonal antibodies

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and the second leading cause of death from cancer in Europe and North America. It is responsible for approximately one million new cases and half a million deaths per year worldwide[1].

Several options are currently available for the treatment of patients with metastatic colorectal cancer (mCRC), including different regimens of chemotherapeutic compounds (fluoropyrimidines, irinotecan and oxaliplatin) and targeted therapies such as bevacizumab and cetuximab. Interestingly, most recent trials that attempt to expose patients to all five drug classes (fluoropyrimidines, irinotecan, oxaliplatin, bevacizumab and cetuximab) target an overall survival (OS) well over 2 years.

In this review we will summarise state-of-the-art targeting of the epidermal growth factor receptor (EGFR) in the management of metastatic colorectal cancer.

BIOLOGY OF EGFR

EGFR belongs to the ErbB family[2]. This family is comprised by transmembrane proteins that form part of the tyrosine kinases receptor proteins which are activated by different kinds of ligands[3] (Figure 1). All the receptor tyrosine kinases share the same protein structure with an extracellular binding domain, a transmembrane domain and an intracellular domain where the catalytic domain is located. The autophosphorylation of tyrosine residues outside the catalytic domain stabilises the receptor in the active conformation and recruit different proteins required for signalling.

There are several ligands binding ErbB including EGF, TGF alpha, Neuregulin family and some others[4]. Not all the ligands 'fit' all the receptors and this feature also has its implications at a molecular level[2]. Once the ligand binds the receptor and the molecule is phosphorylated it can switch on several pathways including the RAS-RAF-MAPK, JAK-STAT and the PIK3-AKT pathways. The signalling pathways activated by different EGF ligands drive various transcription factors to the nucleus that result in different cellular responses such as proliferation, migration, differentiation or apoptosis.

There are four different receptors in the ErbB family named ErbB1 (EGFR; HER or c-erbB the first to be described), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). In the active conformation, the protein forms homodimers or heterodimers that are stabilised by the ligand binding. HER2/neu cannot (due to a genetic mutation) bind to EGF-like ligands and ErbB3 does not have a functional tyrosine kinase.

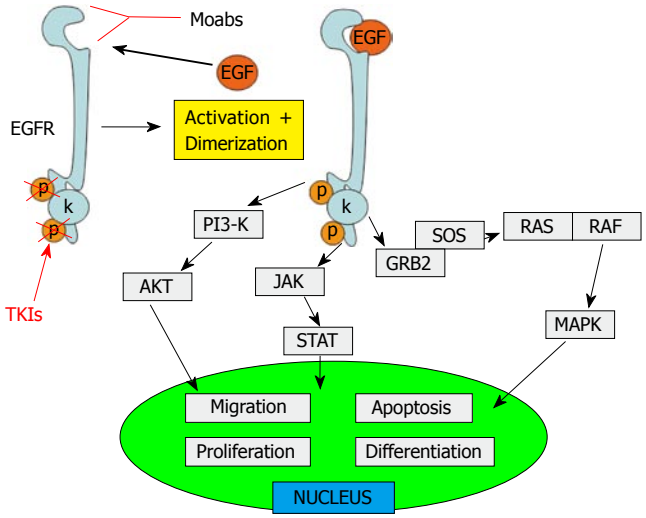

Figure 1 EGFR and its pathways.

Targeting the ErbB network may be achieved by inhibiting the tyrosine kinase (catalytic domain) with small molecules (TKIs) or by inhibiting the extracellular domain with monoclonal antibodies (Moabs) as shown in Figure 1. The moabs block the interaction between natural ligands and the EGF receptor in the extracellular space. The receptor is internalized and that can affect the network, as the timing of this process in the physiological state of the receptor also has its molecular implications[4,5]. Certain antibody isotypes such as IgG1 (cetuximab) have the potential for mediating antibody-dependent cell-mediated cytotoxicity (ADCC) and complement fixation[6], improving thus their antitumor activity. The TKIs compete with the ATP in their binding sites on the catalytic domain of the receptor and so act inside the cell.

CLINICAL APPLICATION

Monoclonal antibodies

Cetuximab: Cetuximab is an IgG1 monoclonal antibody targeting EGFR. Since preclinical data suggested that cetuximab might revert irinotecan resistance *in vitro*[7,8] and *in vivo*[9], a phase Ⅱ study[10] with 121 EGFR expressing mCRC patients refractory to irinotecan was started. A 17% overall response rate (ORR) was documented at an expense of acceptable toxicity grade 3-4. Cetuximab monotherapy has also proved activity in irinotecan refractory patients[11]. A phase Ⅱ open-label clinical trial with 57 EGFR positive mCRC patients was treated and an ORR of 9% was observed. The acne-like skin rash was the main described toxicity related to the drug. Two patients experienced grade 3 allergic reaction and discontinued the study. The study CO.17 that compared cetuximab and best supportive care (BSC) against BSC alone showed that cetuximab provides palliation in pretreated patients with advanced CRC, delaying deterioration in quality of life as well as improving survival[12] (Table 1).

These data led to the design of a study with 329 patients (pts) refractory to irinotecan who were randomized to cetuximab (111 pts) or irinotecan plus cetuximab (CI)

Table 1 Cetuximab in Irinotecan refractory mCRC

	Pts (*n*)	RR (%)	PFS (mo)	OS (mo)
C225 + Innotecan[10]	121	17	-	-
C225[11]	57	9	-	6.4
C225 + Innotecan[13]	329	23	-	8.6

Pts: Patients; mCRC: Metastatic colorectal cancer; RR: Response rate; PFS: Progression free survival; OS: Overall survival; mo: Months; C225: Cetuximab.

(218 pts). The ORR was 22.9% (95% CI: 17.5% to 29.1%) in the CI arm as opposed to 10.8% (95% CI: 5.7% to 18.1%) in the cetuximab arm. OS (8.6 mo *vs* 6.9 mo) and time to progression (TTP) (4.1 mo *vs* 1.5 mo) also favoured the CI arm. The toxicity presented in the CI group was very similar to that of patients treated with irinotecan alone[13] (Table 1).

More mature data regarding the role of CPT-11 and cetuximab in irinotecan refractory patients have been recently reported in the MABEL trial[14]. A multicenter study with 1461 CPT-11 refractory mCRC EGFR positive patients, 64% of whom had received two or more chemotherapy lines; 1123 patients are currently evaluable and a 12-week overall progression free survival (PFS) rate is 61% (58%-64%), and 34% (31%-37%) at 24 wk. The current estimate of median survival is 9.2 mo (8.7-9.9) with grade 3/4 adverse events being diarrhea (20%), skin toxicity (including acne-like rash) (19%), neutropenia (9%) and asthenia (8%). Hypersensitivity reactions occurred in 1.5% of the patients.

The above mentioned results provided the rationale for the BOND2 study that compared the combination of irinotecan, bevacizumab and cetuximab against bevacizumab plus cetuximab in CPT-11 refractory mCRC patients. A 43% ORR as opposed to 27% in favour of the irinotecan arm was presented. The median time to progression was 7.1 mo *vs* 4.6 mo and the median survival was 18.0 mo *vs* 10.3 mo for the irinotecan group[15,16]. The toxicity observed was the expected for each agent alone.

A variety of preclinical data have suggested activity of cetuximab in oxaliplatin resistant tumors[17]. Thus, a phase Ⅱ trial that combined CAPOX (oxaliplatin 85 mg/m^2, d 1, and capecitabine 2000 mg/m^2, d 1-7, every 2 wk) plus Cetuximab in patients who had progressed to oxaliplatin-based regimens has recently been presented[18]. Eighty percent of the 40 patients had also progressed on prior irinotecan-based chemotherapy. The study achieved 1 complete response (CR) (2.5%) and 7 partial responses (PR) (17.5%) with a 20% ORR and a 47.5% disease control rate (DC). The median TTP was 3 mo and the median survival 10.7 mo. Toxicity included grade 3-4 neutropenia (12.5%) and diarrhea (7.5%) and grade 2-3 neurotoxicity (22.5%). The second trial named EPIC is a phase Ⅲ study comparing cetuximab plus irinotecan and irinotecan as a second line in EGFR positive patients who received oxaliplatin plus fluoropyrimidines as a first line therapy. The primary endpoint was overall survival and quality of life being one of the secondary endpoints. Cetuximab plus irinotecan (n = 648) was superior to irinotecan alone (n = 650)

regarding progression-free survival and response rate (16.4% *vs* 4.2%, $P < 0.0001$). OS was comparable between both arms, but it may have been influenced by crossover. Health related quality of life was better preserved on the combination arm with less deterioration in symptom scores (pain, nausea, insomnia) and better health status scores[19]. Main toxicity (> 10%) grade 3-4 were neutropenia (30%) and diarrhea (21%). There is also a study by Lenz *et al*[20] analyzing with 346 refractory to irinotecan, fluoropyrimidines or oxaliplatin EGFR positive patients that achieved a RR of 12% with cetuximab monotherapy in patients.

The preliminary promising efficacy seen with C225 in refractory mCRC has prompted its use as front line therapy. In the ACROBAT study 43 EGFR positive mCRC patients were treated with cetuximab plus FOLFOX with a 77% RR, a median survival of 30 mo and a median PFS of 12.3 mo[21]. The study presented by Rosemberg *et al*[22] in 2002 was designed as a phase Ⅱ study with 27 EGFR positive patients that were treated with irinotecan, 5-fluorouracil/leucovorin (IFL) and cetuximab as frontline. They showed a 44% PR rate with another 20% of patients showing minor responses. Twenty-six out of 27 patients presented with rash, but only 19% were grade 3. Another study with a similar chemotherapeutic scheme was presented by Folprecht *et al*[23] in 2005 with a 67% RR and 29% stable disease rate in 20% of whom their liver metastases were resected after treatment. They used high and normal doses of 5-fluorouracil/leucovorin, three out of fifteen patients presented dose limiting toxicity (DLT) in the group of high dose (2000 mg/m^2). A phase Ⅱ study with 23 EGFR positive mCRC patients of whom 22 were assessable for response were treated with FOLFIRI and cetuximab in first line therapy. It showed a 46% PR rate and a 41% SD rate with a median TTP of 10.9 mo. Most common grade 3/4 toxicities were diarrhea, neutropenia and rash[24]. Seven patients underwent secondary surgery of metastases. Another study with FOLFOX-6 plus cetuximab in chemo-naive patients showed a preliminary 53% ORR with 3 CR[25]. It was a phase Ⅱ study with 82 mCRC patients showing positive or undetectable EGFR expression. 14 patients discontinued the study due to toxicity and 10% of the patients had grade 4 neutropenia and 2% grade 4 sepsis (Table 2).

More recently, results of the CRYSTAL study, a phase Ⅲ clinical trial that compares FOLFIRI plus cetuximab (arm A) *versus* FOLFIRI alone (arm B) in 1217 mCRC have been presented. The median PFS was significantly longer for arm A compared to arm B [8.9 mo (CI: 8-9.5) for group A *versus* 8 mo (CI: 7.6-9) for group B, $P = 0.036$]. RR was also significantly increased by cetuximab (46.9% *vs* 38.7%, $P = 0.005$). The most common toxicities were neutropenia (26.7% in group A, 23.3% in group B), diarrhea (15.2% and 10.5% respectively) and skin reactions (18.7% and 0.2% respectively)[26]. The OPUS study is a phase Ⅲ clinical trial[27] that randomized patients to FOLFOX or FOLFOX plus cetuximab in chemo-naive patients. Their primary objective was response rate and secondary objectives were PFS, OS, and the R0 resection rate after metastatic surgery of curative intent. The preliminary results showed an RR of 35.7% and 45.6% respectively with 337 patients enrolled at that time. The most common grade 3/4 adverse events were neutropenia (27.6% in A; 31.5% in B), diarrhea (7.1% and 6.0%), leucopenia (7.1% and 5.4%) and rash (9.4% in the cetuximab arm only). The COIN study is a phase Ⅲ trial[28] (804 pts) comparing either continuous chemotherapy plus cetuximab or intermittent chemotherapy with the standard palliative combination. The addition of cetuximab to oxaliplatin-fluoropyrimidine combinations results in increased grade 3/4 toxicities overall and specifically to the gastrointestinal (GI), skin rash and lethargy. Capecitabine combination is associated with more GI toxicity but less neutropenia. Unexpectedly, no hypersensitivity reactions have been seen yet on FOLFOX (with or without cetuximab) (Table 3).

Table 2 Cetuximab as frontline, Phase Ⅱ studies

C225 plus:	Pts (*n*)	RR (%)	PFS (mo)	OS (mo)
FOLFIRI[25]	22	80	10.9	-
FOLFOX-4[22]	43	77	12.3	30
FOLFOX-6[26]	82	53	-	-

Pts: Patients; RR: Response rate; PFS: Progression free survival; OS: Overall survival; C225: Cetuximab.

Table 3 Cetuximab as frontline, Phase Ⅲ studies

C225 plus:	Pts (*n*)	RR (%)	PFS (mo)	OS (mo)
FOLFOX Cetuximab *vs* FOLFOX[28]	337	46.6% *vs* 35.5%	-	-
FOLFIRI Cetuximab *vs* FOLFIRI[27]	1217	46.9% *vs* 38.7%	8.9 *vs* 8.0	-

Pts: Patients; RR: Response rate; PFS: Progression free survival; OS: Overall survival.

Panitumumab: Panitumumab is a fully human IgG2 monoclonal antibody directed against the epidermal growth factor receptor. Several trials have tested its role in pretreated mCRC. The study with 148 mCRC refractory to FOLFOX/FOLFIRI EGFR positive patients treated with panitumumab alone showed a 10% RR with 36% of SD. 90% of the patients appeared with skin rash but only 4% G3[29]. Another study with panitumumab in refractory patients to FOLFOX/FOLFIRI[30] showed benefit for treating those patients with Panitumumab *vs* BSC. They were 463 EGFR positive patients who were assigned to panitumumab or BSC alone. The median progression free survival was 8 wk in the Panitumumab group *vs* 7.3 wk in the BSC group and the mean PFS 13.8 wk *vs* 8.5 wk. The RR was 10% in the Panitumumab group and 0% in the BSC group. The main toxicities were rash, diarrhea and hypomagnesemia. They did not find any advantage in overall survival due to the crossover but it resulted in a 46% reduction in the risk of tumor progression. Another study with 91 mCRC pretreated patients with negative or low EGFR by immunohistochemistry (IHC) showed a 7%-9% PR rate with 36%-42% of DC presenting skin and hypomagnesemia as main toxicities[31] (Table 4).

Table 4 Panitumumab, Phase Ⅱ and Ⅲ studies

	Pts (*n*)	RR (%)	PFS	Naive	Phase
Alone[30]	148	10	-	No	Ⅱ
Alone *vs* BSC[31]	463	10	8 wk	No	Ⅲ
Alone[32]	91	8	8 wk	No	Ⅱ
IFL + Panitumumab *vs* FOLFIRI + Panitumumab[33]	19	46	5.6 mo	Yes	Ⅱ
	24	42	10.9 mo		

Pts: Patients; RR: Response rate; PFS: Progression free survival; OS: Overall survival; mo: months; BSC: Best supportive care.

Panitumumab showed better tolerability combined with FOLFIRI than with IFL[32]. In a pooled analysis of several trials[33] the skin toxicity in panitumumab patients was 90%-95% but only in 3%-5% was grade 3 and treatment limiting. The other relevant toxicities were gastrointestinal (nausea, diarrhea and anorexia) which accounts for 25%-30% of all grades (2% grade 3) and hypomagnesemia (41%; 7% grade 3). The severity of skin rash was correlated with increased efficacy in terms of ORR, PFS, and OS[34,35]. A recent study with panitumumab has correlated skin toxicity with increased efficacy and better health-related quality of life[34]. In this phase Ⅲ study patients were randomized to panitumumab plus BSC (231 patients) or BSC alone (232 patients) and the skin toxicity was analyzed in relation to PFS and OS. The incidence of grade 2-4 skin toxicity was higher in the panitumumab arm. OS was significantly prolonged in patients with more severe skin toxicity (gr 2-4 *vs* gr 1; HR = 0.67; $P = 0.0235$) (Table 4).

Tyrosine kinase inhibitors

Gefitinib: Gefitinib is a potent, specific EGFR tyrosine kinase activity inhibitor. Phase Ⅰ/Ⅱ trials in patients with mCRC showed little activity[36,37] but preclinical studies *in vitro* and *in vivo* suggested a supra-additive growth inhibitory effect of gefitinib when combined with different cytotoxic drugs[38] which gave support to several clinical trials of gefitinib combined with chemotherapy in mCRC patients.

The study by Magné *et al*[39] support studies that combined gefitinib with fluoropirimidines[40]. The study was designed in two parts with 23 patients overall. One part with intermittent dose-escalated gefitinib plus 5-fluorouracil (370 mg/m^2 Ⅳ)/LV (20 mg/m^2 Ⅳ) and the other with continuous gefitinib at the safest dose assigned by part one. The safest dose assessed was 500 mg/d achieving a 23% OS with skin rash and diarrhea as main toxicities. Preliminary results from a small phase Ⅰ/Ⅱ trial combining gefitinib 250 mg/d plus capecitabine 1000-1250 mg bid. after failure to first line therapy also suggests some evidence of activity[41].

A dose-finding trial was performed with irinotecan plus gefitinib in 18 patients with advanced CRC refractory to fluoropyrimidine-based chemotherapy. It defined irinotecan given at a dose of 225 mg/m^2 as a single agent every 3 wk plus gefitinib at a dose of 250 mg/d as the maximum tolerated dose (MTD) of this regimen[42]. Dose-limiting toxicities, such as neutropenia and diarrhea, occurred at unexpectedly low doses of irinotecan. Disease stabilization was achieved in 21% (4 out of 18 patients). Once they achieved the recommended dose level (RDL) they expanded the study to a multicenter one with a total of 27 patients at the RDL with an objective tumor response rate of 11% and median survival 9.3 mo[43]. The toxicity grades 3-4 included diarrhea (35.9%), lethargy (15.4%), neutropenia (15.4% with 10.3% febrile neutropenia) and skin rash (7.7%).

The combination of gefitinib plus FOLFIRI in both chemo-naive mCRC patients[44] and as salvage therapy[45] was considered too toxic despite dose reduction in 5-fluorouracil, leucovorin and irinotecan. Toxicity was also the main issue when combining gefitinib with capecitabine in patients who had previously received one or two chemotherapy lines being diarrhea and neutropenia, the principal related DLTs[46].

In a study by Kuo *et al*[47] with 27 patients who had previously received at least one regimen (oxaliplatin based mainly) they employed FOLFOX-4 and gefitinib at a dose of 500 mg/d. 33% of the patients achieved objective responses and 48% showed stable disease. Median OS was 12.0 mo, while median event-free survival was 5.4 mo. For first-line treatment, a 74% RR with a clinical benefit rate of 98% and a median TTP of 9.5 mo. was reported by Zampino *et al*[48] with the FOLFOX-6 regimen plus gefitinib at a dose of 250 mg/daily.

The study by Zeuli *et al*[49] assessed the doses of gefitinib (250 mg/d) plus capecitabine (2000 mg/m^2 per day, d 1-15) and oxaliplatin (120 mg/m^2 d 1) every 3 wk for six courses as first-line treatment in patients with metastatic disease. The most common grade 3 adverse events were diarrhea and neutropenia. A 50% response rate (6 out of 12 patients; 5 PRs, 1 CR) and a clinical benefit rate of 58% (7 out of 12 patients) were communicated.

In an *in vitro* study working with cetuximab-resistant cell lines, authors observed that gefitinib or erlotinib retained the capacity to inhibit growth of tumor cells that were highly resistant to cetuximab[50]. These data suggest that tyrosine kinase inhibitors may further modulate intracellular signalling that is not fully blocked by extracellular anti-EGFR antibody treatment. A phase Ⅰ/Ⅱ study that combined cetuximab and gefitinib[51] presented 56% of PR in mCRC patients. This observation deserves further evaluation.

Erlotinib: Erlotinib is a small molecule that competes with ATP for the intracellular tyrosine kinase domain of EGFR, thereby inhibiting receptor autophosphorylation and blocking downstream signal transduction (Figure 1). Evidence of single agent erlotinib activity *in vitro* and in mCRC patients, derived from disease specific phase Ⅱ studies[52,53], led to the design of several trials in combination with chemotherapy. One phase Ⅱ study presented a PR rate of 4% in 51 mCRC patients. 46 of them were assessed for response. Skin rash was observed in 62% of the patients (13% G3) and grade 3 diarrhea and nausea were also observed after erlotinib monotherapy. Another phase Ⅱ study on 38 mCRC patients treated with 150 mg of erlotinib in a continuous daily schedule presented a 39% SD rate, as the best response, with rash and diarrhea as the main toxicity events[53]. Additive activity of erlotinib when combined with

capecitabine in preclinical studies with human xenografts[54] supported a phase Ⅱ study with 10 pts evaluating the combination of erlotinib 150 g daily with capecitabine 1000 mg/m^2 bid. for 14 d in chemotherapy-naive metastatic CRC patients. Grade 3 diarrhea (30%), grade 3 renal insufficiency (10%) and grade 3 hyperbilirubinemia (10%) were the most troublesome toxicities. Regarding efficacy, no complete responses were achieved whereas disease control rate (PR + SD) was 34%[55].

In the study by Meyenhart *et al*[56] when combining oxaliplatin, capecitabine and erlotinib patients started receiving 1000 mg/m^2 bid. of capecitabine that was reduced to 750 mg/m^2 bid for 14 d after the first 13 patients experienced excess of grade 3/4 toxicities. Thus, the final doses were capecitabine 750 mg/m^2 bid. for 14 d, oxaliplatin at 130 mg/m^2 on d 1, and erlotinib 150 mg daily. The ORR was 20%. In addition, the group of Delord *et al*[57]presented a dose-finding study establishing erlotinib 100 mg/d, capecitabine 1650 mg/m^2 qd (d 1-14), and oxaliplatin130 mg/m^2 every 3 wk as the MTD for this regimen.

Erlotinib (50-150 mg/d) is also being investigated in combination with FOLFOX-4 for untreated or minimally pretreated patients with CRC, with a preliminary reported 43% response rate. The most commonly communicated grade 3 or 4 toxicities were diarrhea and neutropenia[58].

CLINICAL AND MOLECULAR MARKERS OF RESISTANCE AND RESPONSE TO EGFR INHIBITORS

A peculiar toxic effect of cetuximab is a papulopustular skin rash, generally on the face and upper torso, which is thought to be mechanism- and dose-related[59]. Findings suggest that there is a correlation between intensity of skin rash and response and survival[13]. This correlation is particularly striking in a subgroup analysis from the IMC 0144 trial reported by Pippas *et al*. In that trial, patients with no skin toxicity presented no objective responses and had a median survival of 1.7 mo, whereas those who experienced grade 3 skin rash had a 20% RR and a median survival of almost 1 year[60]. This is the first reported observation of a clinical feature that may predict the clinical outcome of an antitumor agent. Dose-escalation schedules are currently under investigation in order to explore the possibility of increasing cetuximab efficacy by inducing skin rash.

The EVEREST study was designed as a phase Ⅲ trial with cetuximab escalated-doses. They started with standard dose and increased dose every 2 wk until skin toxicity grade 2 or 500 mg/m^2 of cetuximab were achieved. The dose-escalation of up to 500 mg/w indicated improvement of RR in pts with no or slight skin reactions on standard dose treatment[61] with 166 patients included in the study. The mechanism underlying the correlation between skin toxicity and tumour response is currently unclear, however, some research groups hypothesized that the rash is a surrogate indicator of an adequate degree of receptor saturation by cetuximab. If this is the case, targeting doses to achieve a desired level of cutaneous toxicity may further increase the efficacy of this agent. While this is an appealing prospect from a potential efficacy point of view, it would suggest, if true, that there might be a narrow therapeutic window when working with this drug[59].

In early clinical trials, EGFR positivity on tumor specimen by IHC was mandatory for the use of cetuximab. However today, EGFR expression status is known not to be a predictive factor of response to cetuximab since major responses in patients with EGFR negative tumors are expected after cetuximab treatment. In fact, responses have been reported by some authors[62] and nowadays EGFR status is not mandatory for the management of CRC patients[63]. Several factors might explain this apparent discrepancy, such as low sensitivity of IHC, cytological heterogeneity of CRC and differential EGRF expression in primary and metastatic tumor niches[64,65]. There are other reasons that might explain these striking data. Two distinct EGFRs have been identified in A431 cells by epidermal growth factor-binding studies. These are a major class of low-affinity EGFR (representing approximately 95% of the receptors) and a minor class of high-affinity EGFR (representing approximately 5% of the receptors), with binding affinities differing by an order of magnitude[66-68]. The current EGFR IHC detection systems used today derived from A431 cells do not distinguish between these two distinct EGFRs. It is known that high-affinity EGFRs are the biologically active receptors that switch the ErbB pathway whereas low-affinity receptors do not contribute significantly[66,69]. Another possible explanation is related to the ADCC capacity of cetuximab antibodies and two polymorphisms related to fragment C of the immunoglobulin G that are related to progression and survival[70].

In order to assess response to EGFR inhibitors in the clinical practice different molecular approaches are being evaluated. There are some studies where they try to find a correlation between some germinal polymorphisms involved in angiogenesis, the EGFR pathway, DNA repair and drug metabolism[15,71]. In a recent study they found a correlation, in patients treated only with cetuximab, between a Cyclin D1 polymorphism (A870G) and overall survival[72]. The Cyclin D1 is a protein related to p27^{KIP1} which is involved in the G1 phase arrest produced by EGFR inhibitors and that is correlated to apoptosis in tumor biopsies of patients treated with gefitinib[73]. The heterozygous AG genotype was significantly related to higher overall survival. Patients with AA homozygous genotype survived a median time of 2.3 mo (95% CI 2.1, 5.7) compared to those having homozygous GG genotype that survived a median of 4.4 mo (95% CI 1.8, 9.8). Even patients with a heterozygous AG genotype presented in comparison, a median survival of 8.5 mo (95% CI 5.5, 11.7), ($P < 0.05$)[72]. Another study showed similar results finding a correlation between EGFR (G497C GA), Cox-2 (G-765C CC) and EGF (A61G GG) polymorphisms and PFS[74].

Furthermore, a different investigation treated mCRC patients with cetuximab or panitumumab assessing the

EGFR copy number and the mutation profile of the EGFR catalytic domain and of selected exons in KRAS, BRAF, and PIK3CA[75] in the tumor sample. They found that in 8 out of 9 patients with an objective response the EGFR copy number was increased whereas only 1 out of 21 non-responders had an increased EGFR copy number. A retrospective study showed a linkage between EGFR mRNA levels by RT-PCR and TTP but not with survival[76] and found no correlation between any other ErbB receptors or EGFR by IHC and clinical outcome. There are other studies that suggested a correlation of KRAS mutation and poor outcome in terms of response and survival[77-79]. In the study by Finocchiaro *el al*[77] they analyzed tumor blocks from 85 colorectal cancer patients for EGFR expression (IHC and FISH), HER2 (FISH) and KRAS (mutation). EGFR FISH positive patients (41 patients) had a significantly higher RR and TTP than EGFR FISH negative individuals (44 patients). EGFR expression assessed by IHC was not associated with any clinical endpoint. Increased HER2 gene copy number predicts early escape from cetuximab therapy. Compared to patients with wild type KRAS, KRAS mutation carriers (32 patients) had a significantly lower RR (6.3% *vs* 26.5%, $P = 0.02$), shorter TTP (3.7 mo *vs* 6.3 mo, $P = 0.07$) and shorter survival (8.3 mo *vs* 10.8 mo, $P = 0.2$). In 22 patients with available primary and metastatic tumor samples, there was no difference between these sites for EGFR FISH, HER2 FISH and KRAS results. A study of 59 mCRC patients treated with cetuximab plus chemotherapy looked for KRAS mutations using first direct sequencing and two sensitive methods based on SNaPshot and PCR-ligase chain reaction (LCR) assays. They compared clinical response with gene mutations. No KRAS mutation was found in the 12 patients presenting clinical response. On the contrary KRAS mutation was associated with disease progression ($P = 0.0005$) and TTP was significantly decreased in patients with mutated KRAS tumors (3 mo *vs* 5.5 mo, $P = 0.015$)[78].

The other important mutations associated with the activity of EGFR inhibitors that are related to response to TKIs in lung cancer are mutations in exons 18, 19 and 21[80,81]. In mCRC it seems not to be the case. That may be due to the fact that those mutations are not commonly found in mCRC patients[20,82,83]. Because of this issue other predictive factors of response to Gefitinib such as the insulin receptor isoform A are currently under research[84].

FUTURE DIRECTIONS IN EGFR TARGETING

Monoclonal antibodies

EMD 72000: EMD 72000 (Matuzumab) is a humanized IgG1 anti-EGFR MoAb. It has completed phase I clinical testing in EGFR-positive solid tumors. 22 patients of different origin (including colorectal) received EMD 72000 weekly[85] and a 23% RR was demonstrated. EMD 72000 administered to 22 patients with colon (15 patients), gastric, or renal tumors demonstrated PR in 2 patients and a minor response in 1 patient[86] all of them with colon cancer. Another phase I study showed near-complete EGFR signalling suppression at the 1200 mg dose level[87]. A phase I study of matuzumab administered weekly to 26 patients (18 of which had CRC) showed 2 PR, and 10 SD in patients with colon cancer. In addition a preliminary analysis of skin biopsies showed that matuzumab produced inhibition of pEGFR and pMAPK with a decrease in Ki67 expression and an increase in p27[88].

AEE788: AEE788 is an oral inhibitor against EGFR, ErbB2, VEGFR-2 and KDR. A phase I study in these patients with advanced CRC and liver metastases showed the lack of clinical activity of AEE up to 400 mg with an inhibitory effect of 100%, 90% and 39% over pEGFR, pMAPK and Ki67 respectively by IHC in tumor biopsies[89]. Another study that investigated the effects of AEE *in vitro* and in biopsies from 22 advanced colorectal cancer patients did not find any major clinical responses even at the higher dose schedule (400 mg). Laser scanning cytometry quantitative analysis confirmed the target inhibition of AEE *in vitro* and in wound-induced skin pairs[90]. The lack of significant target inhibition in tumors has to do with the lack of clinical activity of AEE in this cohort of patients and is consistent with other studies.

HKI-272: HKI-272 is an irreversible pan-erbB receptor tyrosine kinase inhibitor. It inhibits the growth of tumor cells that express erbB-1 and erbB-2 (HER-2) in culture and in xenografts. HKI-272 also inhibits the growth of cultured cells that contain sensitizing and resistance-associated EGFR mutations[91]. A phase I study with 73 patients is ongoing and the preliminary results for 51 patients (3 of which are mCRC) showed a MTD of 320 mg/d with diarrhea as the DLT. Two breast cancer patients had confirmed partial responses and 2 had unconfirmed PRs[92].

Other MoAbs directed against EGFR have recently undergone clinical testing e.g., hR3[93] and ICR62[94].

NEW GENERATION OF TYROSINE KINASE INHIBITORS

Additional oral TKIs currently under clinical evaluation, include the reversible dual EGFR/Her-2 TKI lapatinib and the irreversible EGFR TKI EKB-569.

Lapatinib: Lapatinib is a reversible inhibitor of ErbB1/ErbB2 tyrosine kinases. 64 patients (22 with colon cancer) were included in a phase I study. One CR and 22 SD were achieved. Most of the patients with SD overexpressed either ErbB1 or ErbB2. The most frequent toxicities presented were rash, diarrhea, nausea/vomiting, fatigue, and anorexia. Serum VEGF may be a potential biomarker for lapatinib activity[95]. A study in combination with FOLFOX-4 to assess the safety included 13 patients (2 colon). The dose of lapatinib 1500 mg/d with FOLFOX-4 was well tolerated although 2 patients had grade $\geqslant$ 3 hematological toxicities, which resolved after delay of the next cycle. Seven patients were evaluable for response and 2 PR, 2 SD and 3 PD were confirmed[96]. A phase Ⅱ study with lapatinib as the single-agent in 86 mCRC patients who progressed to prior therapy showed 5 patients who experienced clinical benefit with stable disease

for ⩾ 20 wk[97]. The median TTP and overall survival were 8 and 42.9 wk respectively. The most commonly encountered adverse events were diarrhea (45% grade 1-2, 5% grade 3), rash (33% grade 1-2, 2% grade 3), fatigue (27% grade 1-2, 2% grade 3), nausea (20% grade 1-2, 1% grade 3), anorexia (16% grade 1-2, 2% grade 3), and vomiting (14% grade 1-2).

EKB-569: EKB-569 is a selective, irreversible inhibitor of the EGFR, was well tolerated in patients with advanced solid tumors of the colon, lung, breast, head and neck. A phase I study with 30 patients with advanced tumors of different origins established the MTD at 75 mg EKB-569 per day for both cohorts, intermittent-dose schedule (14 d of a 28-d cycle) and continuous-dose schedule (each day of a 28-d cycle) being the DLT grade 3 diarrhea[98]. In a phase Ⅰ/Ⅱa study of EKB-569 in combination with FOLFOX-4 (29 patients), 4 out of 11 patients who completed 4 cycles achieved a PR, 6 patients had stable disease, and 1 patient had progressive disease[99]. Grade 3/4 Toxicity included neutropenia and diarrhea. Moreover, a phase Ⅰ/Ⅱa study of EKB-569 in combination with FOLFIRI (39 evaluable patients out of 47) showed a 38% of RR[100].

CONCLUSIONS

When administered alone new targeted therapies have demonstrated activity in different *in vitro* and *in vivo* studies. However, the clinical use in patients when administered as a single agent is not so brilliant. On the other hand the combination of these drugs with classical chemotherapies has shown better clinical profiles reflected in an improvement in OS and PFS. The FDA approved Cetuximab as a second line therapy in combination and Panitumumab has also been approved as a second and third line therapy for advanced CRC patients. An important number of clinical trials with second or first generation of TKIs is ongoing. Perhaps the role of TKIs in mCRC patients is maintenance treatment in individuals with objective response or stabilisation of their tumor.

There is also the challenging possibility of combining different targeted therapies in order to overpass tumor resistance. Combining targeted therapies against different pathways is also a possibility. The cross-talk at a molecular level of the different networks implicated in cell biology is almost unknown. However there are more data that implicate different molecular networks when studying resistance to targeted therapies against one pathway.

All these data must encourage clinicians and basic researches to hold on in their efforts of untangling the network behind EGFR trying to transform all that effort in improving patients quality of life as well as improving survival There are different clinical scenarios in our patients and each of them should have its own solution. In some cases the approach will be combining chemotherapy with targeted therapy, targeted therapy with radiotherapy or even targeted therapy alone. In anyway we have still a lot of clinical trials to start and new drugs to be tested in order to find the adequate solution for each of our patients.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Pharmacogenomics in colorectal cancer: The first step for individualized-therapy

Eva Bandrés, Ruth Zárate, Natalia Ramirez, Ana Abajo, Nerea Bitarte, Jesus García-Foncillas

Eva Bandrés, Ruth Zárate, Natalia Ramirez, Ana Abajo, Nerea Bitarte, Jesus García-Foncillas, Laboratory of Pharmacogenomics, Cancer Research Program (Center for Applied Medical Research), University of Navarra, Navarra, Spain
Correspondence to: Eva Bandrés Elizalde, PhD, Laboratory of Pharmacogenomics, Center for Applied Medical Research, University of Navarra, Avda Pio XII 55, Pamplona 31008, Spain. ebandres@unav.es
Telephone: +34-948-194700 Fax: +34-948-194714
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Abstract

Interindividual differences in the toxicity and response to anticancer therapies are currently observed in practically all available treatment regimens. A goal of cancer therapy is to predict patient response and toxicity to drugs in order to facilitate the individualization of patient treatment. Identification of subgroups of patients that differ in their prognosis and response to treatment could help to identify the best available drug therapy according the genetic profile. Several mechanisms have been suggested to contribute to chemo-therapeutic drug resistance: amplification or overexpression of membrane transporters, changes in cellular proteins involved in detoxification or in DNA repair, apoptosis and activation of oncogenes or tumor suppressor genes. Colorectal cancer (CRC) is regarded as intrinsically resistant to chemotherapy. Several molecular markers predictive of CRC therapy have been included during the last decade but their results in different studies complicate their application in practical clinical. The simultaneous testing of multiple markers predictive of response could help to identify more accurately the true role of these polymorphisms in CRC therapy. This review analyzes the role of genetic variants in genes involved in the action mechanisms of the drugs used at present in colorectal cancer.

Key words: Colorectal cancer; Pharmacogenomics; Chemotherapy; Polymorphisms; Markers

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INTRODUCTION

Colorectal cancer (CRC) is the second most prevalent cancer and the third leading cause of cancer death worldwide with almost 500 000 related deaths every year[1]. Approximately half of all persons develop local recurrence or distant metastasis during the course of their illness, and the median survival time for these patients can vary from approximately 4 to 22 mo. The basis of treatment for metastasis or recurrent colorectal cancer is chemotherapy, although small number of patients can undergo surgery or others forms of loco regional treatment. While the Dukes and Tumor Node Metastasis (TNM) staging system identifies broad patients groups that vary in their long-term prognosis, considerable heterogeneity exists within each of different chemotherapy agents with regard to response to treatment.

The most studied drug in CRC, the antimetabolite 5-fluorouracil (5-FU), was developed over 40 years ago. In the metastasis disease setting, single-agent 5-FU produced response rates of only 10%-20%[2]. Over the last 5 years, the median survival for patients with metastasis colorectal cancer has nearly doubled from 12-22 mo and the combination of 5-FU with new classes of drugs, such as oxaliplatin and CPT-11 (Irinotecan), has significantly improved response rates up into the 40%-50% range in patients with metastasis colorectal cancer[3]. Figure 1 shown chemical structure of these compounds. Furthermore, the use of novel biological agents, such as the monoclonal antibodies Cetuximab (an epidermal growth factor receptor (EGFR inhibitor) and Bevacizumab (a vascular endothelial growth factor (VEGF) inhibitor), have recently been shown to provide additional clinical benefit for patients with metastatic colorectal cancer[4,5].

The objective of pharmacogenomics is to elucidate the complex genetic network responsible of drug efficacy and adverse drug reactions. The ultimate goal is to provide new strategies for optimizing the individual's response to drug therapy based on patient's genetic information[6]. Current methods of basing dosages on weight and age will be replaced with dosages based on an individual's genetics. This will maximize the therapy's value and decrease the likelihood of overdose.

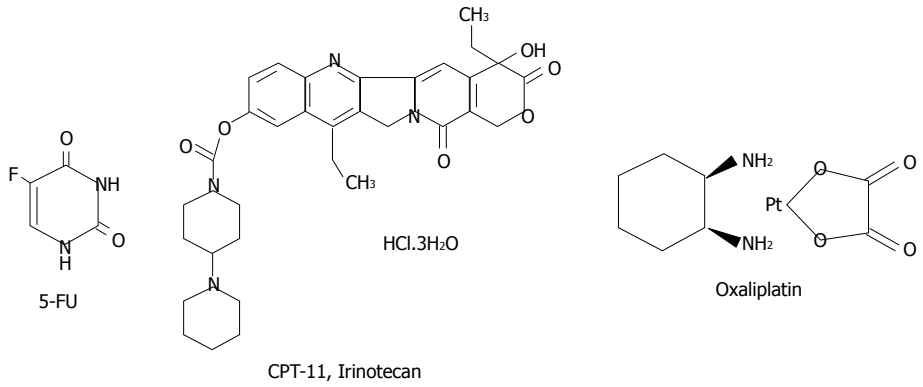

Figure 1 Chemical structure of the three most important drugs used in colorectal chemotherapy: 5-FU, CPT11 and Oxaliplatin.

In CRC, a limited number of predictive markers have been identified to date. The use of these as individual predictive markers has led to somewhat conflicting results. However, if these markers are used in combination they could provide a greater ability to reliably predict response to treatment[7]. Recent advances in our understanding of the molecular biology of CRC should lead to the identification of other panels of potential prognostic and predictive markers.

POLYMORPHISMS AND FLUOROPYRIMIDINES

To this day, the fluoropyrimidines (FPs) including 5-fluorouracil (5-FU), 5'-fluoro-2'-deoxyuridine, capecitabine, tegafur and S1, remain a major component of many standard regimens for numerous cancer types and a baseline component in many experimental regimens with novel agents[8]. Initially, 5-FU was the only effective systemic treatment for CRC, and since leucovorine enhances this effect, 5-FU and LV are given together[9]. FL reduces tumor size by 50% or more in approximately 20% of patients with advanced CRC, and prolongs median survival from approximately 6 mo to approximately 11 mo. When given as adjuvant therapy after the complete resection of tumor that has spread to regional lymph nodes (Stage Ⅲ), FL increases the probability of remaining free of tumor at 5 years from approximately 42% to 58% and the likelihood of surviving for 5 years from 51% to 64%[10].

5-FU, an analog of uracil, is an anticancer prodrug that, after administration, is converted intracellular into three main active metabolites: 5-fluoro-2-deoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The main toxic effects are mediated by the inhibition of thymidylate synthase (TS) through the formation of an extremely stable ternary complex among FdUMP, TS, and the cofactor 5, 10-methylene-tetrahydrofolate (CH_2FH_4)[11]. The formation of this complex prevents the methylation of the deoxyuridine -5'-monophosphate (dUMP) into deoxythymidine-5'-monophosphate (dTMP) catalyzed by TS. However, the incorporation of the FP metabolites, FdUTP and FUTP, into DNA and RNA respectively, contribute also to 5-FU cytotoxicity[12] (Figure 2).

The common role played by FPs makes stratification according to likely response to this agent a relevant starting point in efforts to individualize treatment. For this purpose, reliable indicators for the prediction of the expected response are required. In the last few decades, intensive research aimed at understanding FP activity and extensive testing of patient's outcomes have highlighted a number of characteristics as potential indicators of response.

Overexpression of TS has been reported in many types of tumors including breast, colon, gastric, and melanoma. In particular, TS overexpression has been found to be significantly associated with a low response to treatment based on 5-FU, both as adjuvant[13] and metastatic therapy[14] Several studies have proposed that genetic polymorphisms of TS gene can affect the response to 5-FU[15-17]. TS expression seems to depend on the number of the so-called TSER, tandem repeat polymorphic copies of 28 bp present in the 5'-promoter enhancer region of the gene[18]. TSER polymorphisms, therefore, are involved in the modulation of TS protein levels and can affect the drug response after administration of fluoropyrimidine. Most Caucasian subjects may be carriers of double (TSER*2) or triple (TSER*3) repetitions for this type of polymorphism, although there have also been reports of sequences with even more copies. An increase in the number of repeats gives rise to an increase in both mRNA and protein TS levels. Three copies of such repeats (TSER*3) lead to a TS expression which is 2.6 times higher than that produced by the presence of only two copies (TSER*2). Patients with CRCs, which show homozygote triple-tandem repeats (3R/3R), present high levels of intratumoral TS mRNA, elevated levels of TS protein, and a lower rate of response to chemotherapy than subjects with CRCs showing homozygote double-repeats (2R/2R)[19]. Similar results have been obtained in patients with metastatic CRCs[20]. Moreover, a study involving 221 Duke's C stage CRC patients has shown that, with regard to survival rate, tumors with 3R/3R genotypes benefit less from chemotherapy than those with 2R/2R and 2R/3R genotypes[16]. A meta-analysis of 20 studies has made it possible to investigate the association between levels of TS expression and the survival of CRC patients[21]. The results have shown that high levels of TS in patients at any stage of the disease are predictive of outcome[22]. However, the predictive role of TS levels in early-stage CRC patients

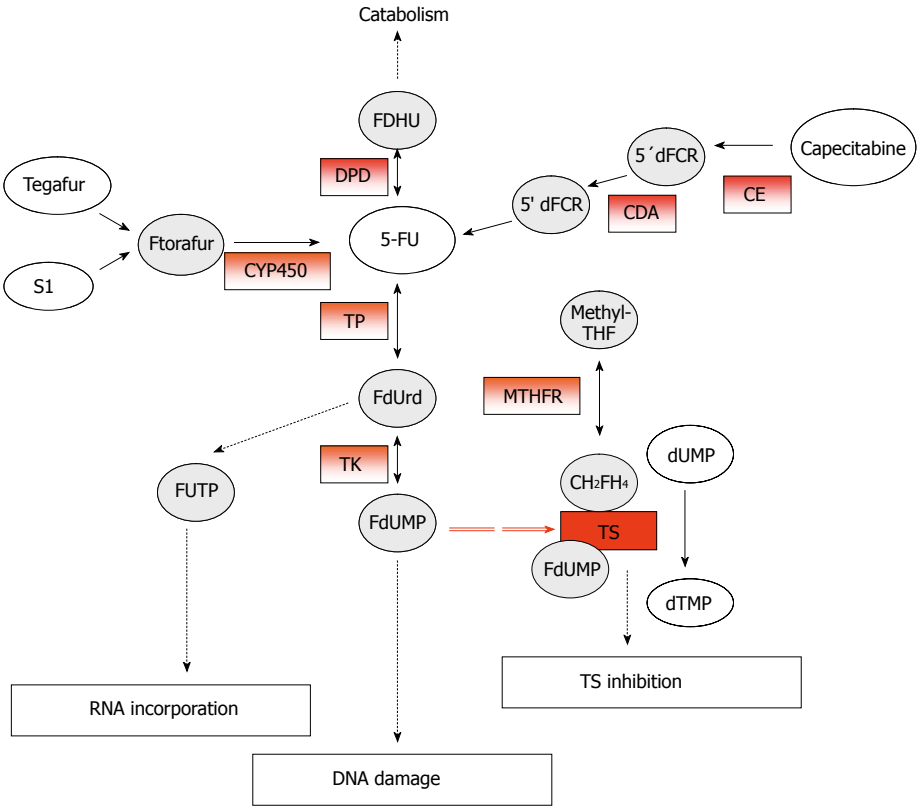

Figure 2 Metabolism and mechanism of action of 5-fluoruracil (5-FU). The potential predictive markers for 5-FU response are in red-boxes.

undergoing chemotherapy is still not fully understood; in fact, whereas in subjects undergoing surgery only, high TS levels are an independent prognostic factor for outcome, in those undergoing surgery and adjuvant FU, TS expression does not seem to predict outcome. Another study reports that in patients with advanced CRC treated with 5-FU/oxaliplatin, intratumoral TS levels appear to have an independent predictive value for survival[23]. Nevertheless, the data so far reported in literature are discordant; although, in fact, TS levels have prognostic value for CRC, this is lower in surgically-treated patients who undergo adjuvant therapy with 5-FU when the TS expression is low, but may be effective for tumors with high TS expression.

TP, also known as platelet-derived endothelial cell growth factor, catalyzes the conversion of 5-FU to the more active nucleoside form and has been shown to be an *in vitro* determinant of 5-FU activity. High expression of either TS or TP in colorectal tumors was shown to be an independent variable so that low expression of both enzymes in tumors predicted a very high expression rate to 5-FU as well as a significantly longer survival, whereas none of the patients with high expression of either TP or TS were responders. These data are in contrast to those demonstrating that cells with higher levels of TP should be more sensitive to 5-FU. These discrepancies may be due to the fact that high TP gene expression was not directly reflected in its protein products, and 5-FU metabolism may be limited by the availability of co substrates, or due to the role of TP as an angiogenic factor.

5-FU is inactivated in the liver by dihydropyrimidine dehydrogenase (DPD), which is the first key enzyme involved in the catabolism of the uracil and thymine into β-alanine. DPD activity is extremely variable in tumoral tissue and this variation might make a difference to the efficiency of 5-FU treatment, since intratumoral drug concentration is one of the most important factors for the determination of the antitumoral effect[24]. Deficiency in DPD activity, however, leads to severe toxicity correlated to 5-FU which may even be fatal. The partial or total lack of this enzyme has, in fact, been associated with severe toxicity (mucositis, granulocytopenia, and neuropathy), and in several cases even death, after 5-FU administration[25]. Analysis of the prevalence of various genetic variants of DPD among patients with DPD deficiency has shown that the most common mutation in DPYD is a G-A transition at the invariant GT splice donor site flanking exon 14 (IVS14 + 1G > A) in Caucasian populations; this mutation is responsible for the lack of exon 14 in mRNA transcript resulting in production of a truncated mRNA with virtually not present enzyme activity[26]. This allele is known as DPYD*2A and is one of the variants associated with severe toxicity after 5-FU treatment[27]. Recently two new missense mutations have been identified on codon 496 (A→G) in exon 6 and on codon 2846 (A→T) in exon 22, the latter in a patient with a total lack of DPD[28].

In the last few years, with the recognition that CH_2FH_4 was essential for the formation of the FdUMP-TS ternary complex, folate metabolism has also begin to emerge as a focus for FP response prediction. MTHFR converts CH_2FH_4 to 5-methyltetrahydrofolate. Consequently, it could be expected that the functionally comprised C677T variant would lead to increase CH_2FH_4 concentrations and thereby enhanced FP activity. Further support of a role for folate metabolism in determining FP response has been provided by the observation of a survival benefit from 5-FU treatment for colorectal cancer patients with DNA

hypermethylation. Higher levels of folate intermediates, including CH_2FH_4, have been demonstrated in tumors with DNA hypermethylation[29]. Cohen and colleagues[30] found a statistically significant trend towards increased response to fluoropyrimidine-based chemotherapy with increasing copy number of the MTHFR 677 T allele in a study of 43 patients with metastatic colorectal cancer. In contrast, Wisotzkey and co-workers[31] did not observe a difference in survival by MTHFR C677T genotype among 51 Stage Ⅲ colon cancer patients treated with 5-FU. However, both studies had a small number of subjects with the MTHFR 677TT genotype (n = 5), and lacked adjustment for potential confounding factors such as primary tumor site or type of chemotherapy received. Only one study has evaluated the effects of the MTHFR C677T, A1298C and TSER genotypes on time to progression and response to 5-FU-based treatment. Jakobsen and co-workers[32] studied 139 patients with metastatic colorectal cancer being treated in a randomized trial comparing three different 5-FU dosage levels. A greater percentage of individuals with the TSER 3R/3R or MTHFR 677T genotypes responded to treatment, and these same individuals had a statistically significant increase in time to disease progression for the first 8 mo post-treatment. However, later in the course there was no statistically significant difference in time to relapse by MTHFR or TS genotype.

Treatment of metastatic CRCs now includes the use of another chemotherapeutic agent, Capecitabine, which is an oral precursor of 5-FU. Due to its poor bioavailability and rapid catabolic clearance by DPD, 5-FU is unsuitable for oral delivery. Capecitabine or Xeloda® is a rationally designed oral fluoropyrimidine carbamate that, after selective conversion to 5-fluorouracil within solid tumors, acts by inhibiting thymidylate synthase activity. This would theoretically yield two advantages, enhanced drug concentrations at the tumor site and thus greater antitumor activity, and reduced drug levels in normal tissues with a consequent reduction in systemic toxicity.

Capecitabine is well absorbed by the gastrointestinal tract and undergoes a three-step enzymatic conversion to 5-FU. First metabolized in the liver by carboxylesterase to 5'-deoxy-5-fluorocytidine, capecitabine is converted in the liver and tumours tissues by citidine deaminase to 5'-deoxy-5-fluorouridine. A tumor-selective phenomenon is facilitated by higher intra-tumoral levels of thymidine-phosphorilase, the enzyme responsible for the final conversion step to 5-FU. With regard to 5-FU, low levels of TS and DPD lead to a better response to capecitabine. In particular, it has been observed that 75% of metastatic colorectal cancer patients, with homozygote double-repeat variants in TS (2R/2R), respond better to capecitabine administration compared with 8% of those with hetero-zygote variants (2R/3R) and 25% of those with triple-repeat homozygote variants (3R/3R)[33].

Recent advances in our understanding of the molecular biology of CRC should lead to the identification of other panels of potential prognostic and predictive markers associate with colorectal carcinogenesis.

In CRC, genetic instability has been recognized as a factor in the origin of malignant lesions, resulting in clonal evolution of genetic events acquired in the course of tumor progression. Microsatellite instability (MSI) is common to many forms of cancer and is found in 10%-14% of sporadic colon cancers[34]. MSI is caused by mutations in the mismatch repair (MMR) genes, such as hMSH2, hMLH1 and hMSH6, resulting in failure of the DNA MMR system to correct errors that occur during replication. An *in vitro* study[35] demonstrated that restoration of hMLH1 activity in the MMR-deficient HCT116 cells increased their sensitivity to 5-FU. Various studies have investigated the prognostic role of MSI in Stage Ⅱ CRC. The studies have confirmed a consistent and independent association between MSI-high (MSI-H) phenotype and superior survival in Stage Ⅱ and Stage Ⅲ CRC patients[36]. Furthermore, Lim *et al*[37] demonstrated that patients with MSI tumors exhibited better recurrence-free survival compared with those with microsatellite stable (MSS) tumors. Moreover, the use of adjuvant chemotherapy did not benefit these patients. The use of MSI as a predictive marker of response to adjuvant chemotherapy still remains controversial. On the other hand, it has been reported that 70% of colorectal cancers have lost a portion of chromosome 17p, or 18q or both. The 17p chromosome contains the p53 gene, which is an important tumor suppressor, and is reported to be mutated in 40%-60% of colorectal cancers[38]. p53 status has been studied as a prognostic factor, and more recently as a predictor of response to cancer chemotherapy[39]. The study published by Tang and colleagues describe that p53 mutation was associated with a poorer prognosis in Stage Ⅱ and Ⅲ CRC patients who received surgery alone, whereas p53 was not a prognostic factor among those patients who had received 5-FU-based adjuvant chemotherapy[40]. However, Ahnen and co-workers found that patients with Stage Ⅲ CRC, whose tumors overexpressed p53, did not derive significant survival benefit from adjuvant 5-FU-based treatment[41].

POLYMORPHISMS AND IRINOTECAN

The combination of 5-FU together with other drugs such as Irinotecan (CPT-11) has led to promising results in the treatment of CRCs, particularly in first line therapy of patients with metastatic disease. Partly as a result of the development of this agent, survival of patients suffering from incurable colorectal cancer has doubled during the last decade[42]. Like other camptothecins, the anti-neoplastic agent irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) and in particular its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin) stabilize the DNA-topoisomerase I complex by binding to it, preventing the resealing of single strand breaks[43]. Irinotecan prevents the replication division to proceed which results in double strand breaks and ultimately in its anti-tumor effect and its characteristic adverse effects on rapidly dividing tissues, such as bone marrow and intestinal mucosa. The main dose-limiting toxicities of irinotecan therapy are therefore myelosuppression and delayed-type diarrhea[44,45].

In humans, irinotecan is hydrolyzed into its active metabolite SN-38 by carboxylesterases, present in serum, intestines, tumor tissue, and in high content in

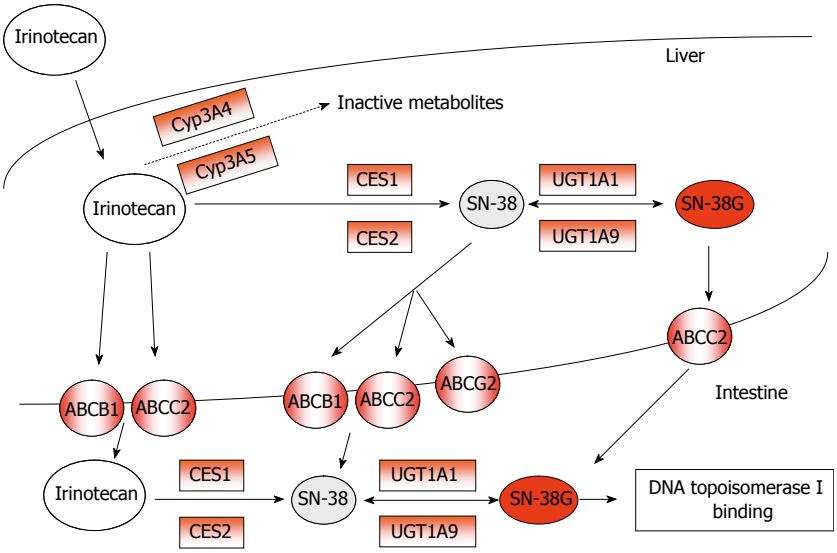

Figure 3 Metabolism and mechanism of action of Irinotecan (CPT-11). The potential predictive markers for CPT-11 response are in red-boxes.

the liver[46]. Recently, the opinion is emerging that intratumoral activation of irinotecan into SN-38 by CES might be even more important than systemic circulating SN-38 levels, formed by hepatic CES[47]. Although plasma levels of SN-38 are relatively low, relations between SN-38 and myelosuppression and/or diarrhea have been demonstrated[48]. Uridine diphosphate-glucuronosyltransferase 1A (UGT1A) mediated glucuronidation of SN-38, forming a β-glucuronic acid conjugate (SN-38G; 10-O-glucuronyl-SN-38), is the main pathway of detoxification for SN-38. Irinotecan is also sensitive to cytochrome P450 3A (CYP3A) that mediated oxidative pathways, resulting in the formation of inactive metabolites. Moreover, irinotecan, SN-38, and their metabolites are excreted by drug-transporting proteins from the adenosine-triphosphate binding cassette (ABC) transporter superfamily[49] (Figure 3).

The CES genes, located on chromosome 16q13-q22, are supposed to be highly conserved during evolution. However, recently, several polymorphisms in the CES-genes have been described, some of which with major racial differences in distribution[50]. Although the interpatient variation in CES activity is high and some SNPs appear to be very common[51], the functional consequences of reported SNPs on the *in vivo* activation of irinotecan into SN-38 are thought to be limited. Marsh *et al*[50] did not demonstrate any functional relationship between the presence of SNPs in the CES genes and CES mRNA levels, except for an intronic SNP (IVS10-88) in CES2 which was associated with reduced CES2 mRNA expression in colorectal tumors, but not in normal colonic mucosa. Neither did Charasson *et al*[52] find any influence of 11 silent SNPs in CES2 on gene expression or functional activity. Lack of association may be explained by the ineffective activation of irinotecan by CES, the role of other esterases, and the complex metabolic pathway of irinotecan. It may also be possible those other proteins regulate CES transcription and translation, or that other factors are rate limiting in the formation of active CES. However, as SNPs in CES may lead to less transcription and thus might lead to diminished local activation of irinotecan and less favorable therapeutic responses, both *in vitro* and *in vivo* functional investigation of SNPs in the CES genes is needed, especially of recently discovered SNPs in CES2.

Members of the cytochrome P450 superfamily are capable to oxidize more than half of all anti-cancer drugs. Especially the CYP3A subfamily, and in particular, the genes CYP3A4, CYP3A5, CYP3A7, and CYP3A43 are the most important. CYP3A4*1B, a SNP in the promoter area of the gene, was thought to be a promising polymorphism for irinotecan pharmacokinetics, partly as a result of its relatively high allele frequency compared to most other CYP3A4 SNPs[53]. However, Garcia-Martin *et al*[54] reported that the presence of CYP3A4*1B did not correlate with low enzyme activity in Caucasians. In a polygenetic approach to assess genotypes from multiple irinotecan pathway genes with irinotecan pharmacokinetics no effect on irinotecan pharmacokinetics was seen, neither for this SNP nor for the other studied CYP3A SNPs (CYP3A4*2, CYP3A4*3, CYP3A5*3 and CYP3A5*6)[55].

The human UGT superfamily has been classified into the UGT1 and UGT2 families, further classified into three subfamilies (UGT1A, UGT2A, and UGT2B)[56]. All nine functional members of the UGT1A subfamily are encoded by a single gene locus, the UGT1A locus on chromosome 2q37. Especially the UGT isoforms 1A1, 1A7 and 1A9 are involved in the phase Ⅱ conjugation of SN-38 to the inactive metabolite SN-38G[57]. UGT1A1 and UGT1A9 are highly expressed in the gastrointestinal tract and the liver; the primary organ involved in the detoxification of irinotecan. Polymorphisms, resulting in absent or very low UGT1A1 activity, have been associated with three heritable unconjugated hyperbilirubinemia syndromes: Crigler-Najjar syndrome type 1 and 2[58], and Gilbert's syndrome[59]. Gilbert's syndrome is common among Caucasians and is associated with the presence of an extra, seventh, dinucleotide (TA) insertion (UGT1A1*28) in the $(TA)_6$TAA-box of the UGT1A1 promoter region, leading to a considerable reduced enzyme expression of about 30%-80%. The UGT1A1 activity appears to be inversely related to the number of TA-repeats, varying from 5 to 8.

Studies have shown that the homozygous UGT1A1*28 genotype was associated with an increased risk of developing leucopenia and severe delayed-type diarrhea after treatment with irinotecan. Ando *et al*[60] analyze the association between UGT1A1 variants and irinotecan toxicity, revealing in a multivariate analysis that presence of UGT1A1*28 allele was a risk factor for severe toxicity. These data have been confirmed by other groups[9,61]. Based on this knowledge and the finding that demonstrated a good concordance between the UGT1A1*28 genotype and less effective SN-38 glucuronidation prospective studies were initiated. A significant relation was observed between the AUC of SN-38 and the number of TA-alleles[62]. In addition, two other promoter variants (UGT1A1-3279G>T and UGT1A1-3156G>A) have been identified. These variants are in strong linkage disequilibrium with the UGT1A1*28 polymorphism in Caucasians, while this link is less apparent in African-Americans and Asians, suggesting a different haplotype structure among various races[63]. Ando *et al*[64] found a strong a relation for presence of the UGT1A1-3263T>G SNP and the severity of irinotecan induced toxicity, although in a multivariate analysis including UGT1A1*28 as well, this effect was mainly attributed to this latter polymorphism[65]. Presented observations clearly illustrate that UGT1A1 mutations can influence a patient's exposure to SN-38, and, hence, the susceptibility to toxicity. Recently, a study in colorectal cancer cell lines shown that DNA methylation represses UGT1A1 expression and that this process may contribute to the level of tumoral inactivation of the anticancer agent SN38 and potentially influence in clinical response[66].

The adenosine-triphosphate (ATP) binding cassette (ABC) transporters are the largest family of transmembrane proteins that use ATP-derived energy to transport various substances over cell membranes[67]. Their localization pattern suggests that they have an important role in the prevention of absorption and the excretion of potentially toxic metabolites and xenobiotics, including irinotecan and its metabolites.

P-glycoprotein, located on chromosome 7q21, and, among others, expressed in kidney, liver, and intestine, is known for more than 50 SNPs and other polymorphisms in the gene encoding this transporter[68]. Three SNPs which show linkage disequilibrium (ABCB1 1236C>T, ABCB1 2677G>A/T, and ABCB1 3435C>T), have been studied extensively [69]. However, a relation with irinotecan or its metabolites has been not demonstrated in Caucasians. Recently, Balram *et al*[70] showed a relation for ABCB1 3435C>T with irinotecan AUC (area under concentration versus time curves) in a small Chinese population which may be the result of lowered pump activity. In a group of 46 Caucasian patients, a significant effect of the ABCB1 1236C>T polymorphism on the AUCs of irinotecan and SN-38 was seen, resulting in an increase in both AUCs[71]. Although an effect of these three related SNPs on irinotecan pharmacokinetics seems likely, the true clinical relevance of their effects still remains to be clarified.

For the canalicular multispecific organic anion transporter (ABCC2), recently a functional SNP in irinotecan pharmacokinetics has been found (ABCC2 3972C>T). This SNP, studied in 64 Caucasian patients, resulted in highly significant effects on the AUC of irinotecan, and SN-38G, all being higher in patients carrying two 3972T alleles.

In vitro studies have indicated that the irinotecan metabolites SN-38 and its glucuronide conjugate SN-38G are very good substrates for the breast cancer resistance protein[72]. ABCG2, located on chromosome 4q22, was first found to be overexpressed in cancer cells with acquired resistance to anticancer drugs[73]. The ABCG2 gene is supposed to be well conserved and most SNPs found up to now seem unlikely to alter transporter stability or function[74]. Few SNPs with presumed clinical consequence have been studied in relation to irinotecan pharmacokinetics; in particular, a single-nucleotide polymorphism in exon 5 has been described. This ABCG2 421C>A transversion results in an amino acid change of glutamine to lysine at codon 141[75]. Functional consequences of this SNP were demonstrated in Caucasian cancer patients treated with the structurally related camptothecins diflomotecan and topotecan[76]. Patients carrying at least one defective ABCG2 421A allele were found to have higher drug levels. However, in a large group of Caucasian patients pharmacokinetic parameters of irinotecan and SN-38 were not significantly different[77].

POLYMORPHISMS AND OXALIPLATIN

Oxaliplatin (OXA), a third-generation platinum analog that distorts DNA adducts, administered alone or in combination with 5-FU/LV has broaden the therapeutic choices for patients with advanced CRC who may experience hepatic and pulmonary metastasis. The cytotoxic activity of oxaliplatin is initiated by formation of a DNA adduct between the adequated oxaliplatin derivative and a DNA base[78]. Initially, only monoadducts are formed but eventually oxaliplatin attaches simultaneously to two different nucleotide bases resulting in DNA cross-links. The adducts are formed with the N-7 positions of guanine and adenine preferentially and in most cases these reactions result in intrastrand cross-links. In the cell approximately one of every 100 000 bases can be cross-linked by a platinum atom, resulting in 10 000 platinum atoms per cell[79].

In general, the cytotoxic efficacy of platinum compounds in cancer cells can be related to inhibition of DNA synthesis or to saturation of the cellular capacity to repair Pt-DNA adducts. Platinum atoms modify the three-dimensional DNA structure, which inhibits the normal DNA synthesis and repair processes[80].

Interestingly, cellular DNA repair mechanisms seem to differ in their response to Pt or Pt-DACH complexes. After DNA-adduct formation by oxaliplatin, cells will activate cellular repair mechanisms. In general, DNA repair is carried out by specific enzymes that consist of several amino- and sulphur groups. Therefore, oxaliplatin can be covalently bound to these repair enzymes as well, impairing their function[81]. If substantial DNA damage persists this may ultimately lead to the activation of apoptotic pathways and cell death[82].

Several mechanisms are described that confer resistance to oxaliplatin, including diminished cellular drug

accumulation, increased intracellular drug detoxification and increased Pt-DNA adduct repair. However, the overall sensitivity of a cell is multifactorial and the relative importance of each process on ultimate drug sensitivity is difficult to predict[83]. There is growing evidence that common gene variants affect the activity of cellular DNA repair and platinum conjugation.

The uptake of platinum by cells is not completely understood but there is evidence that decreased accumulation is the most common mechanism of resistance to cisplatin[82]. Platinum uptake by cells is an energy requiring process, but it is not saturable and possibly involves transport by a yet unidentified efflux pump. Once inside the cell, conjugation to glutathione (catalyzed by the enzyme glutathione-*S*-transferase, GST) effectively inactivates platinum compounds before DNA damage is induced. This conjugation reaction is followed by cellular excretion and is therefore related to cellular drug resistance as well. A number of studies indicate an important role of GST in oxaliplatin resistance. A single nucleotide polymorphism (SNP) in exon 5 at position 313 (A→G) in the GSTP1 (π) gene results causes the amino acid change Ile105→Val. The mutant GSTP1 (π) enzyme is less potent in detoxification of carcinogens and individuals with two mutant alleles have shown a significant survival benefit from combined oxaliplatin/5-FU treatment[84]. Other common polymorphisms in the GSTT1 (θ) and GSTM1 (μ) genes include deletions that result in complete loss of enzyme activity in homozygous individuals. However, no association with altered survival or clinical response in patients with advanced colorectal cancer treated with oxaliplatin/5-FU was observed for the GSTT1 and GSTM1 genotypes[85].

Since the primary anti-tumor mechanism of oxaliplatin is the formation of Pt-DNA adducts, polymorphisms in genes involving the repair of these adducts, such as nucleotide excision repair, base excision repair, mismatch repair (MMR) and other post-replicative repair pathways, may affect oxaliplatin efficacy. Induction of the enzymes involved in these systems results in increased DNA repair activity, more efficient adduct removal and hence decreased sensitivity to platinum drugs.

Mismatch repair (MMR) is a DNA repair pathway that corrects base mispairs and small strand loops that occur during replication. Loss of MMR function results in an increased spontaneous mutation rate. The MMR system consists of six different proteins, originating from the hMLH1, hMLH2, hPMS2, hMSH2, hMSH3 and hMSH6 genes. *In vitro* studies showed that MMR is not involved in oxaliplatin induced DNA-damage repair, whereas it serves as an important mechanism in cisplatin and carboplatin adduct repair[86]. The conformational distortion of the oxaliplatin DNA complex is different from the cisplatin and carboplatin adduct and this, together with the less polar properties of the DACH-ligand, contributes to a recognition failure of MMR proteins to detect oxaliplatin adducts. To date, no polymorphisms in the MMR pathway genes are known that influence the anti-tumor effects of oxaliplatin.

Single-strand breaks resulting from exposure to endogenously produced active oxygen, ionizing radiation or alkylating agents are repaired by the base excision repair system. X-ray repair cross-complementing group 1 enzyme (XRCC1) contains a domain which functions as a protein-protein interface that interacts with poly (ADP-ribose) polymerase (PARP). Shen *et al*[87] identified three polymorphisms in the XRCC1 gene. One of these, located in exon 10 of this gene, causes the amino acid change Arg399→Gln in the PARP binding domain. The polymorphic enzyme is supposed to be less capable of initiating DNA repair due to altered binding characteristics. In individuals with the mutant Arg399→Gln codon increased DNA damage marker levels are found due to inadequate repair or increased damage tolerance. Patients with at least one of the mutant alleles have a more than five old risk of combined oxaliplatin/5-FU chemotherapy failure compared to patients with two wild type alleles[88].

Nucleotide excision repair is a pathway involved in the recognition and repair of damaged or inappropriate nucleotides. A wide variety of DNA-damage is repaired by NER, including UV-induced photo-products, helix-distorting monoadducts, cross-links and endogenous oxidative damage. At least six proteins are essential for damage recognition and removal by this repair pathway. The first step in this process is recognition of a damaged or inappropriate base by XPA (xeroderma pigmentosum complementation group A protein) and RPA (replication protein A). The adhesion of XPA and RPA to a DNA strand attracts other repair factors to the site followed by enzymatic unwinding of the helix lesion area by XPD. The XPD gene, also known as ERCC2 (excision repair cross complementing group 2), encodes an ATP-dependent helicase that is a component of transcription factor TFIIH. A significant relationship with clinical response to platinum-based chemotherapy was found for the Lys751 →Gln polymorphism of ERCC2[89]. This SNP causes an amino acid change in exon 23 and apparently affecting protein function but not resulting in an alteration of any of the seven helicase domains. Metastatic colorectal cancer patients treated with oxaliplatin/5-FU showed different tumor response for the various genotypes; 24% responders in the Lys/Lys group, *versus* 10% in the Lys/Gln and 10% in the Gln/Gln groups, respectively[90]. Nevertheless, further studies are necessary in order to confim these data and to establish the real importance of polymorphisms in the gene XPD with regard to resistance to platinum agents.

TARGETED-THERAPIES FOR COLORECTAL CANCER

Targeted therapy is defined as a treatment with a focused mechanism that specifically acts on a well-defined target or biological pathway. The ideal cancer target can be defined as a macromolecule that is crucial to the malignant phenotype and is not expressed significantly in vital organs and tissues bind to cancer cells with high affinity and create anti-tumor effects.

In colorectal cancer, two targets, the process of angiogenesis, and the epidermal growth factor receptor, are exploited by the newest monoclonal antibodies that are available for use in CRC patients (Figure 4).

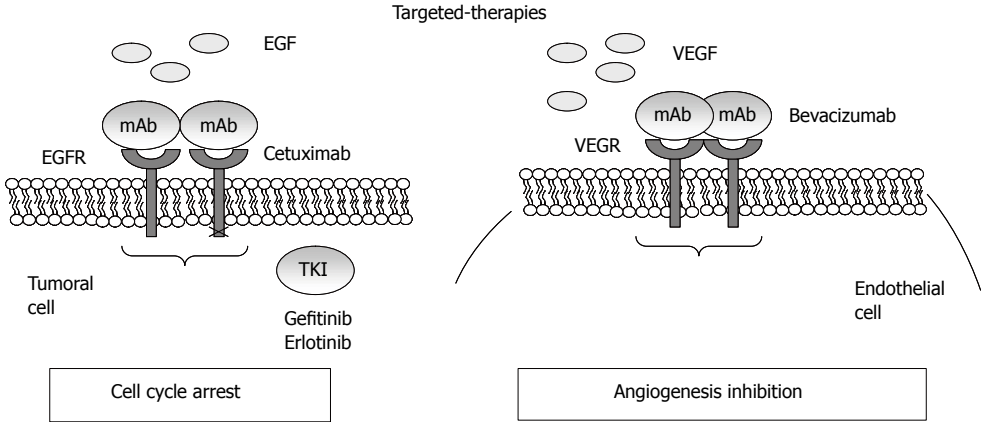

Figure 4 Mechanisms of action for the epidermal growth factor receptor and VEGF.

EGFR-based therapies

EGFR is a tyrosine kinase receptor of the ErbB family that is abnormally activated in epithelial tumors, including 25%-80% of CRCs[91]. EGFR is a 170-kDa cell surface glycoprotein containing three well-identified parts: an extracellular binding domain, a hydrophobic membrane-spanning domain and a cytoplasmic domain containing the tyrosine kinase activity. The bind of specific ligands, EGF and TGFα, to the extracellular domain, leading to dimerization of the receptor with another EGFR (homodimerization) or another member of the EGFR family (heterodimerization). Its activation leads to downstream signaling that stimulates mitogenic and survival pathways such as mitogen-activated protein kinases (MAPKs) and phosphotidylinositol-3 kinase (PI3K)/Akt, which have tumor-promoting activities. Inhibition of these signaling pathways by EGFR antagonists can lead to induction of Bax, activation of caspase-8 and downregulation of Bcl-2 and NF-κB, initiating a cascade of intracellular signaling that ultimately regulates cell proliferation, migration, adhesion, differentiation, and survival[92,93]. Tumor cells that may be activated by ligands such as EGFR and TGFα may then become chemosensitive through EGFR inhibition and activation of these apoptotic pathways.

Agents targeted against the EGFR have been studied extensively in the laboratory, and several have undergone clinical trials, including Cetuximab (Erbitux), a humanized monoclonal antibody directed against the extracellular domain of the EGFR, and the small molecule tyrosine kinase inhibitors (TKIs) Gefitinib (Iressa/ZD1839), and Erlotinib (Tarceva/OSI-774).

Cetuximab binds to the EGFR with high affinity, blocking growth-factor binding, receptor activation, and subsequent signal-transduction events[94]. Preclinical models demonstrated modest *in vitro* and *in vivo* single-agent activity of Cetuximab but significant enhancing activity in combination with cytotoxic chemotherapy[95]. Cetuximab enhanced the antitumor effects of chemotherapy and radiotherapy by inhibiting cell proliferation, angiogenesis, and metastasis and by promoting apoptosis[92]. Several studies have shown that cetuximab is effective in patients with metastasic CRC whose disease has progressed on irinotecan-based chemotherapy. A phase Ⅱ study of cetuximab monotherapy in EGFR-positive advanced CRC patients that failed a previous treatment with irinotecan, obtained 10.5% partial responses and disease stabilization in 35% patients[96]. The result of a multicenter phase Ⅱ study in 246 advanced CRC patients that failed two lines of chemotherapy containing fluoropyrimidines, oxaliplatin and irinotecan have confirmed a partial response of 12% and a disease stabilization rate of 34%. The most important data for the use of cetuximabb, was derived from a large European randomized study, the BOND study, which compared cetuximab with cetuximab in association with irinotecan. Partial response were obtained in 22.9% patients treated with irinotecan plus cetuximab and the time of disease control was 55.5 mo[4].

The development of cetuximab in colorectal cancer was grounded on the premise that EGFR expression by IHC would be prognostic for cetuximab activity, with all trials to date requiring EGFR positivity by IHC. However, Chung *et al*[97] demonstrate no correlation between intensity of EGFR expression and clinical response, challenging this premise. The BOND study results, obtained similar conclusion and the probability or achieving a response was not correlated to the level of EGFR expression in the tumor[4]. On this basis, EGFR-negative colorectal cancer patients would not be excluded from standard protocol treatment with cetuximab on the basis of EGFR status. EGFR analysis by current IHC techniques does not appear to have predictive value, and selection or exclusion of patients for cetuximab therapy on the basis of currently available EGFR IHC does not appear reasonable[98]. This may be due in part to the lack of a standardized protocol and grading system for EGFR expression in clinical samples to technical limitations that are inherent in immunohistochemical methods or, perhaps, to an intrinsically poor correlation between the level of EGFR expression and therapeutic response.

A polymorphic (CA)n dinucleotide repeat is observed in intron 1 of the EGFR gene, which has been shown to be associated with gene expression[99]. It has been demonstrated that as the number of (CA)n repeats increases the level of transcription decreases[100]. However, in CRC cancer, association between the repeat length and EGFR protein expression was not been reported[101]. Neither, polymorphisms of EGFR has been associated with cetuximab therapy.

In addition to cetuximab, several tyrosine kinase

inhibitors have been developed to target EGFR. A recent phase Ⅱ study shown that the combination of capecitabine, oxaliplatin, and erlotinib seems to have promising activity against metastatic colorectal cancer in patients who received prior chemotherapy, with a relatively higher response rate and progression-free survival compared with previous reports of either infusional FU, leucovorin, and oxaliplatin or capecitabine and oxaliplatin in similar patient populations[102].

Skin rash has been the most commonly observed toxicity associated with the various EGFR inhibitors; interindividual differences in the onset, duration and severity of the rash have been observed, and no threshold plasma levels have been linked to the occurrence of the rash. Most intriguing are emerging data demonstrating a significant correlation between skin rash and survival among various patients treated with different anti-EGFR therapies. There are several potential hypotheses being put forward to explain both the variable toxicity and efficacy of EGFR inhibitors. One such hypothesis proposes that variability in clinical observations is related to variable drug exposure. For example, the small-molecule EGFR tyrosine kinase inhibitors gefitinib and erlotinib are metabolized by *CYP3A*, and it is certainly plausible that individuals with variant *CYP3A* alleles might have differences in drug exposure. On the other hand, the previously described CA dinucleotide repeat polymorphism might influence the drug response due to differences in target expression. Data that indirectly lend support to this hypothesis come from a higher response rate observed in Japanese patients compared to Caucasian patients (when treated with gefitinib) two populations with a difference in the frequencies of the *EGFR* dinucleotide repeat variants. However, given the abundant *EGFR* expression in skin tissue, and the observed association between skin toxicity and tumor response; the use of surrogate tissue in this instance might be justified. Nonetheless, this issue highlights an important problem in conducting translational work in this field, since obtaining tumor biopsies in prospective trials for hypotheses generation is not a trivial matter for obvious ethical and practical concerns.

However, robust predictive markers are needed in order to identify the relatively small subsets of patients whose tumours are likely to respond to EGFR-targeted therapies. Candidate markers include phosphorylated EGFR, and phosphorylated effector molecules downstream of the EGFR, such as the mitogen-activated protein kinase (MAPK) and protein kinase B (AKT). However, there are concerns about the stability of phosphorylated proteins in primary tumour samples prior to fixation, and protocols for the collection and processing of clinical material for phosphorylated protein analysis have yet to be validated and standardized. More recently, a work shown that *KRAS* mutation is associated with resistance to cetuximab and a shorter survival in EGFR-positive metastatic colorectal cancer patients treated with this therapy[103]. *KRAS* mutation status might allow the identification of patients who are likely to benefit from cetuximab and avoid a costly and potentially toxic administration of this treatment in nonresponder patients. Prospective randomized study is needed to validate these results that bring a new possibility of targeted therapy adapted to each patient according to its *KRAS* mutation status.

Future issues in the development of EGFR inhibitors include the identification of biologic predictors of response, combination with other targeted agents, and their use in earlier stage malignancies.

VEGF as target for anti-angiogenic therapy

The VEGF family comprises six molecules, the best characterized of which is VEGF-A, which is expressed in at least four isoforms derived by alternative splicing. It is a multifunctional cytokine that acts with receptors expressed on the vascular endothelium to render microvessels hyperpermeable to plasma proteins, alters gene expression, induces endothelial cell migration and proliferation and enhances endothelial cell survival, eventually leading to angiogenesis, permeability and protection against endothelial cell apoptosis and senescence[104,105]. VEGFs mediate their functions by binding to one or more of three tyrosine kinase receptors expressed on endothelial cells: VEGF receptor VEGFR-1 (Flt-1), VEGFR-2 (Flk-1 or KDR) and VEGFR-3 (Flt-4). These receptors have tyrosine kinase activity that initiates intracellular signaling on ligand binding[106]. Other receptors identified (neuropilin-1 and -2) are expressed on numerous cell types, but they do not transmit intracellular signals by themselves after ligand binding[107].

VEGF is a major target for antiangiogenic therapy since its overexpression has been associated with vascularity, endothelial cell migration and invasion, poor prognosis and aggressiveness in most malignancies, including CRC[108]. In CRC, the overexpression of VEGF and its receptor correlated with the development of metastasis[109]. Anti-VEGF strategies include neutralizing antibodies to VEGF or its receptors, ribozymes to receptors and TKIs that block downstream signaling despite ligand binding to VEGFR. Several of these strategies are currently under investigation, including Phase Ⅰ, Ⅱ and Ⅲ trials.

Bevacizumab is a humanized monoclonal antibody that targets and binds to vascular endothelial growth factor-A (VEGF-A), reducing the availability of VEGF and thereby preventing receptor activation[110]. Kabbinavar *et al*[5] reported the first clinical trial of bevacizumab in combination with 5-fluorouracil and leucovorin (5-FU/LV) in previously untreated colorectal cancer patients. Then, different clinical trials shown that Bevacizumab increases survival in association with chemotherapy in the treatment of metastasic CRC. These data led to the FDA approval of bevacizumab for the treatment of metastatic colorectal cancer in February 2004.

As cetuximab, the development of bevacizumab has not included a diagnostic eligibility test and the identification of biomarkers that may predict which patients are most likely to respond to targeted-therapies is of considerable interest. To date, neither direct measurement of VEGF expression nor assessment of tumor microvessel density has been incorporated into the clinical trials or linked to the rates of response to this antibody.

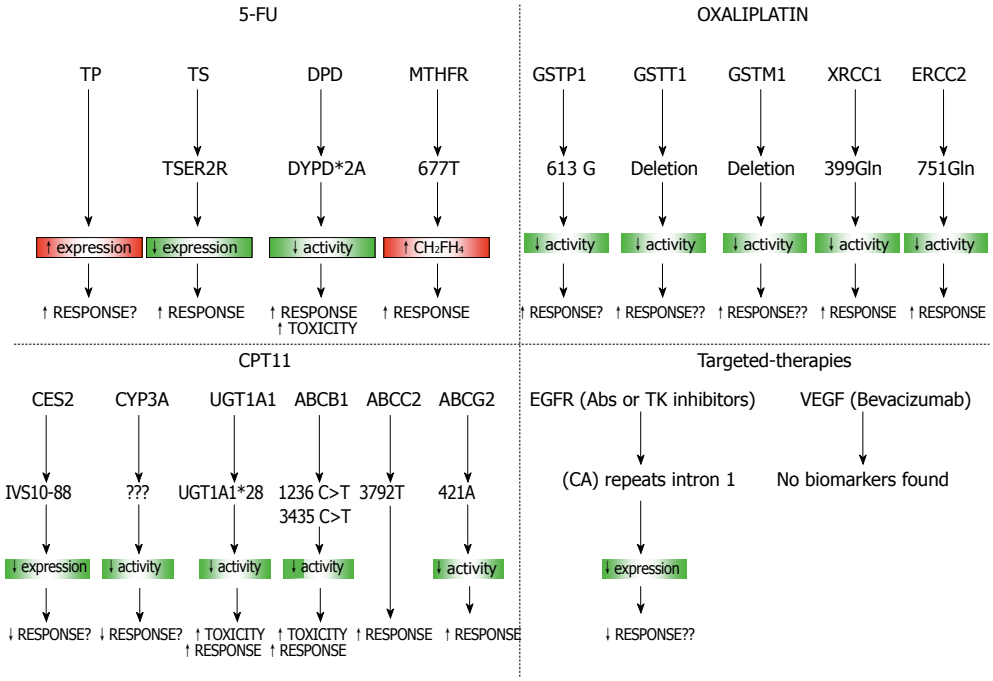

Figure 5 Combination of predictive gene sets for different therapies used in CRC.

Possible biologic surrogates which have been tested in some clinical trials include: DCE-MRI, positron emission tomography scan assessment of tumor blood flow[111], mutations in k-ras, b-raf and p53 genes[112], circulating endothelial progenitors, mature circulating endothelial cells[113], or plasma levels of angiogenic markers, e.g.,VEGF, bFGF. To date, few studies have assessed the potential utility of biomarkers in predicting which patients are more likely to respond to antiangiogenic therapy in the clinic. Tumors may express multiple pro-angiogenic factors and, thus, have different pathways to bypass the VEGF inhibition. Likely, biomarkers that summarize the effects of all angiogenic regulators may better predict patient outcome than the analysis of a single angiogenic factor.

FUTURE PERSPECTIVES

Over recent years, a large number of studies have attempted to define molecular and biochemical markers that may be useful predictors of response to treatment. The introduction of DNA microarray technology has revolutionized our approach to understanding the molecular events regulating the drug-resistant, allowing the simultaneous assessment of thousands of genes. This approach provides a valuable means to identify novel biomarkers of response to treatment as well as novel molecular targets for therapeutic intervention. The candidate gene approach has been widely used to identify the genetic basis for pharmacogenetic traits and becomes increasingly more powerful with the recent advances in genomic technologies. The simultaneous testing of multiple markers predictive of response could help to identify more accurately the true role of these polymorphisms in CRC therapy (Figure 5). High-throughput sequencing and SNP genotyping technologies allow the study of thousands of candidate genes and the identification of those involved in drug efficacy and toxicity. Combination of predictive gene sets identified by gene expression profiling with proteomics and SNPs-array methodologies may enhance the prediction of tumor response to chemotherapy and provide further insights into the molecular characterization of tumor cells. In future studies it will important to combine all these technologies to identify the tumoral response to chemotherapy and finally realize an individualized treatment regimen to each patient.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Novel translational strategies in colorectal cancer research

Ignacio Gil-Bazo

Ignacio Gil-Bazo, Cancer Biology & Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, United States
Correspondence to: Ignacio Gil-Bazo, MD, PhD, Department of Oncology, University Clinic, University of Navarra, Pio XII 36, Pamplona 31008, Spain. igbazo@unav.es
Telephone: +34-948-255400 Fax: +34-948-2554003
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Abstract

Defining translational research is still a complex task. In oncology, translational research implies using our basic knowledge learnt from *in vitro* and *in vivo* experiments to directly improve diagnostic tools and therapeutic approaches in cancer patients. Moreover, the better understanding of human cancer and its use to design more reliable tumor models and more accurate experimental systems also has to be considered a good example of translational research. The identification and characterization of new molecular markers and the discovery of novel targeted therapies are two main goals in colorectal cancer translational research. However, the straightforward translation of basic research findings, specifically into colorectal cancer treatment and *vice versa* is still underway. In the present paper, a summarized view of some of the new available approaches on colorectal cancer translational research is provided. Pros and cons are discussed for every approach exposed.

Key words: Translational research; Colorectal cancer; Genomics; Proteomics; Targeted therapies

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INTRODUCTION TO COLORECTAL CANCER

In the current century, despite the recent achievements in the treatment of advanced colorectal carcinoma (CRC), this tumor remains a major public health concern. In fact, it comprises the third most common cancer type to occur in men and women and was the second leading cause of death among cancer patients in the United States during 2006[1].

Different surgical approaches can guarantee low recurrence rates and high survival expectancy in stages I to Ⅲ colon neoplasm patients[2]. Furthermore, adjuvant chemotherapy administration has been shown to effectively improve those rates[3]. However, the subset of stage Ⅱ colon cancer patients to whom adjuvant therapy should be offered is still to be addressed[4]. In fact, different molecular pathology studies and genomic/proteomic investigations are working on that task[5].

In contrast, metastatic colorectal cancer is still far away from being a curable condition and the main goals in the treatment of stage Ⅳ colorectal cancer are to decrease tumor-related symptoms or, alternatively, to prolong symptom-free survival with tolerable toxicity[6,7]. However, the emergence of the highly selective therapeutic antibodies bevacizumab and cetuximab has definitely improved the survival of patients with metastatic CRC[8,9]. This fact has intensively boosted the search for other targeted therapies directed to other fundamental checkpoints in colorectal tumorigenesis[10,11].

Thus, due to colorectal cancer clinical and economic relevance, its basic and clinical research has become one of the most funded among all tumor types in most developed countries. However, the straightforward translation of basic research findings into colorectal cancer therapies is still underway.

In the present paper, a summarized view of some of the new available approaches on colorectal cancer translational research is provided.

TRANSLATIONAL RESEARCH IN CANCER: DEFINING CONCEPTS

Translation of the exciting novel findings made in basic laboratories into testable hypotheses for evaluation in clinical trials is the ultimate aim of translational research in oncology[12-14]. Between a laboratory breakthrough and a real achievement in the clinic, there must be translational research. Thus, the job of the translational researcher is to take the knowledge gained in the laboratory and lay the groundwork needed to develop a new diagnostic tool for a human tumor or a novel drug to be tested in a clinical trial in human beings (Figure 1).

In other words, in order to improve human health,

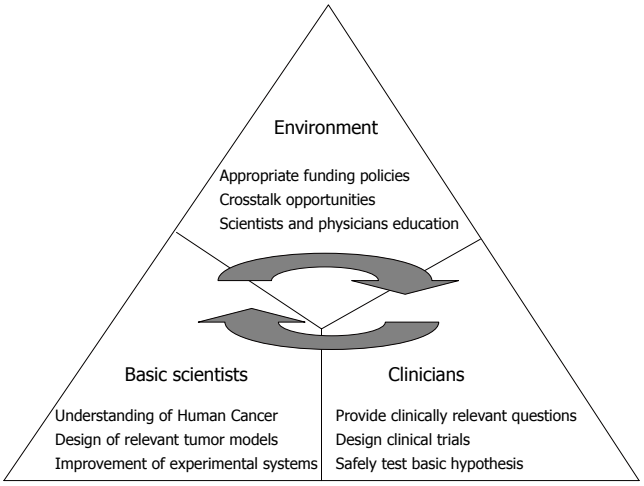

Figure 1 Factors involved in translational research: Interaction between basic scientists, clinicians and the environment.

scientific discoveries must be translated into practical applications. Such discoveries typically start at "the bench" with basic research, in which scientists study disease at a molecular or cellular level[15-19], and then move on to the clinical level, or the patient's "bedside"[20-22]. Scientists are increasingly aware that this bench-to-bedside approach to translational research should really be a two-way highway (Figure 1). Basic scientists provide clinicians with new tools to be used in patients and for assessment of their impact whereas physician-scientists formulate the clinically relevant questions to be tested by basic researchers in a better controlled and more simplified system. Actually, discoveries travel from the clinic to the laboratory in the form of clinical observations, human tissue, diagnostic images, and blood samples, which researchers use to further unlock the molecular and cellular features of cancer (Figure 1).

Often, translational research involves animal studies designed to mimic human conditions[23-26]. Such studies are generally performed with the same care and scrutiny as the best-planned human clinical trials, and comprise a complex set of supporting laboratory techniques that aim to determine how and why the new diagnostic tool or therapy works or fails in these models. Translational research studies may involve many years of investigation on tools and techniques, to try to estimate how safe and how effective the new treatment or diagnostic procedure will be in human trials.

One of the main scopes of translational research in cancer implies the identification and characterization of molecular markers[12]. These can be employed as diagnostic and prognostic tools but also for drug responsiveness assessment or even for targeted therapy design. Molecular markers of tumor responsiveness to drugs would help to select the patient populations that would most likely respond to the drug and identify therapeutic indications. Molecular markers of drug activity in normal tissue would allow pharmacodynamic monitoring of patients that could aid optimization of drug dosing and scheduling to maximize patient response[27]. Furthermore, biological markers involved in tumor initiation and progression can be specifically targeted by new drugs such as therapeutic antibodies[8,9] or anti-tumor vaccines[18].

In fact, another main goal in cancer research is targeted therapy[22]. Translational research is particularly feasible now because of the new understanding of what causes cancer in different individuals, which relates to different combinations of genetic events. This understanding has come primarily from the work of basic research scientists. Until fairly recently, the only effective armamentaria in cancer therapy were surgery, radiation therapy, and chemotherapy. These treatments generally affect neoplastic cells but also non-cancer tissues, leading to the often serious toxicity that characterizes most of traditional cancer treatments[28]. While these standard therapies will continue to play an important role in the treatment of patients with cancer, they can be vastly aided in this process by targeted drugs, which literally target the aberrant molecular pathways that are actually involved in tumor initiation and progression. Therefore, specifically delivering the targeted drug to the malignant cell and its closest environment can significantly relieve cancer treatment related collateral effects[27].

However, since extensive libraries of cytotoxic compounds are being developed for antitumor effect testing, it is becoming more and more common to find new therapies that are successfully developed, tested and commercialized against certain tumors but the ultimate molecular mechanisms involved in tumor response are not clearly known[29]. In those cases, the translational process is rather directed from clinical findings to basic cellular and molecular experiments (from "the bedside" to "the bench"), trying to unravel the complex pathway in which the new compound is playing a definitive role and the specific target or group of them that results inhibited. Therefore, the bidirectional nature of translational research needs to be emphasized[30].

IMPLEMENTING TRANSLATIONAL RESEARCH IN COLORECTAL CANCER

There is still a widening gap between basic research and clinical practice, particularly for colorectal cancer. This might be due to the genetic and molecular complexity of this tumor, the lack of the ideal *in vivo* model for colorectal cancer, and the difficulties found in reproducing animal results into clinical trials in patients.

The principal directions toward which translational research has spread and grown in colorectal cancer in recent years are genomics and proteomics, oncogenic pathways assessment and new targeted therapies discovery (Table 1).

Genomics and proteomics: Searching for new biomarkers and potential target genes

In the last years, there has been an increasingly high effort in the use of genome information in biomedical sciences. This genome information has greatly expanded the insight into the genetic basis of cancer, comprising one of the main fields of interest in translational cancer research. Traditional methods of identifying novel targets involved

Table 1 Translational research technologies in colorectal cancer

Genomics	Proteomics
DNA microarrays	2D-PAGE
	DIGE
	LC-MS/MS
	ICAT
	iTRAQ
	Protein microarrays
	MALDI-TOF
	SELDI-TOF
	Tissue microarrays
Oncogenic pathways	Preclinical models
AS-ODN	Min mice
miRNAs	Msh2, Msh4, Msh6 deficient mice
siRNAs	Apc163 8N mice
	Smad4/Apc mice

in cancer progression were based on studies of individual genes. The following understanding, however, has also shown that gene analysis alone is not sufficient to explain why cancer appears and progresses[31].

Now, the use of DNA microarrays facilitates the analysis of the expression of thousands of genes at the same time and rapidly[32,33]. Microarray analysis has been used for gene expression analysis of different neoplasms[34,35], including CRC[36-39]. However, the application of DNA microarray technology for analysis of CRC is of limited value since it fails to offer direct protein expression measurements[36,40]. In addition, it is already known that important pathways in colon tumorigenesis are regulated at the posttranscriptional level where RNA expression data cannot offer any further information. In fact, due to the alternative splicing of both mRNA and proteins, combined with protein posttranslational modifications, one gene can encode a considerable protein population. Actually, the proteome comprises all proteins that result from the whole genome. In contrast to the genome, the proteome is rather a dynamic parameter constituted by proteins and reflects both the intrinsic genetic program of the cell as well as the impact of its surrounding environment.

However, only a few studies have looked for a further insight into the function and/or importance of individual genes and their application to the proteome research of a tumor. Some of these genes have been proposed as candidate cancer biomarkers[41-43]. More recently a number of proteomic studies have also addressed the identification of potential targets in CRC[44-46].

In the proteomics field, several different technical strategies have been developed and applied to CRC translational research over the last years. Each one has its own advantages and drawbacks that should be considered before deciding the experimental design[47].

The technique leading the field for a long time was the two-dimensional polyacrilamide gel electrophoresis (2D-PAGE)[48]. The 2D-PAGE is based on the separation on a gel of the protein content of a sample in two dimensions according to mass and charge. The gels are stained and spots in samples are compared among different gels. However, a number of serious disadvantages such as its lack of real high-throughput capability (one sample per gel) is responsible for having been replaced by more advanced and capable techniques. Similar to 2D-PAGE, the two-dimensional difference gel electrophoresis (DIGE)[44,46] strengthened the 2D platform by allowing the detection and quantization of differences between three samples resolved on the same gel, or across multiple gels, when linked by an internal standard. Again, it also is a low-throughput technology that does not permit the comparison of many samples in a feasible manner.

Other low-throughput proteomic techniques have recently evolved for cancer protein profiling such as liquid chromatography coupled to tandem mass spectrometry detection (LC-MS/MS)[49], isotope-coded affinity tag (ICAT)[50] and a variation of the latter, isotope tags for relative and absolute quantification (iTRAQ)[51], (both consist of a differential tagging of proteins from samples that are compared using isotope-coded affinity tag in an isotope-dilution mass spectrometry experiment).

A study conducted by Wu *et al*[52] has recently compared some of these diverse proteomic strategies (2D-DIGE, ICAT and iTRAQ) on HCT-116 colon epithelial cells concluding that regarding the number of peptides detected for each protein by each method, the global-tagging iTRAQ technique was more sensitive than the cysteine-specific ICAT method, which in turn was as sensitive as, if not more sensitive than, the 2D-DIGE technique.

Nevertheless, as aforementioned, one of the most important goals in protein profiling in oncology is the discovery of new biomarkers[53]. The use of molecular markers in translational research has expanded considerably during the last 3 decades, and this increased analysis of specific molecular changes has been associated with a concomitant decline in the use of more general and less specific histochemical stains and biochemical assays. Some of the applications for molecular markers include diagnosis, early detection, and prognosis. Also, specific molecular markers are used to study the biology of the disease, to identify targets for novel therapies (e.g., use of Herceptin), and to aid the selection of specific therapies, as previously mentioned.

Therefore, cancer proteomic studies might identify disease-related biomarkers for early cancer diagnosis and new surrogate biomarkers for therapy efficacy and toxicity, but also for guidance of optimal anticancer drug combinations, enabling tailor-made therapy[54]. Furthermore, they could lead to new pharmacological targets. However, a crucial requisite for this purpose is to be able to perform a systematic analysis of a large number of proteins in an easy, reproducible, time-efficient and cost-effective way. High throughput technologies are therefore warranted.

Protein microarrays for instance[55], (targeted proteins bind to spotted probes on a "forward" microarray and specific probes bind to targeted proteins in spotted samples on a "reverse" microarray; bound proteins are detected by direct fluorescent labeling or by labeled secondary antibodies), provide a high throughput approach in terms of number of probes per "forward" array and

number of samples per "reverse" array with the advantage of previously knowing the biomarker identity. On the other hand, the synthesis of many different probes is necessary, the identity of biomarkers has to be known and cross-reactivity of probes along with possible impaired binding of proteins with post-translational modifications (PTM) exists.

In 2002, the Nobel committee acknowledged the advances in mass spectrometry of biopolymers with the recognition of the discovery of electrospray ionization (ESI) mass spectrometry[56,57] and for the discovery of soft laser desorption (SLD) ionization, which led to the development of matrix-assisted laser desorption ionization (MALDI)[58]. These discoveries for peptides, proteins and other macromolecules have been revolutionary, providing easy measurements of molecular weight with unprecedented accuracy. Because the dominant ions generated under SLD and MALDI conditions are singly charged, the technique is most often used in combination with a time-of-flight (TOF) analyzer to extend the m/z range to 100 000 Da and beyond[58]. MALDI-TOF technology is a highly capable tool allowing the measurement of up to 1536 samples per plate, also possessing access to PTM. On the negative side, this technique is unsuitable for high molecular weight proteins (> 100 kDa) and sample fractioning is needed when measuring complex samples.

Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) technology is a variant of MALDI-TOF in which a selected part of a protein mixture is bound to a specific chromatographic surface and the rest is washed away[47]. Although SELDI-TOF technology only permits 96 samples to be tested by bioprocessor, fractioning of the sample is not necessary and direct application of the whole sample is possible. However, compared to MALDI-TOF, SELDI-TOF provides lower resolution and mass accuracy but requires smaller amounts of starting material. SELDI-TOF is also unsuitable for proteins heavier than 100 kDa.

SELDI-TOF is equally useful for the analysis of cell lysates from cell lines and tissue[59], however, in clinical practice its ultimate value derives from its application to easily accessible body fluids as serum or urine. In fact, in the last years several serum biomarker proteins have been identified through this technical approach[60-62].

In addition, low and high throughput techniques have been shown to be complementary and its combination can lead to a more efficient outcome[63].

In summary, compared to the genome, the proteome provides a more reliable picture of a biological status and is, thus, expected to be more useful than gene analysis for evaluating, for example, disease presence, progression and response to treatment.

A totally different approach for protein profiling has recently emerged in translational cancer research. To evaluate the clinical significance of newly detected potential cancer genes, it is usually required to examine a high number of well-characterized primary tumors. Using traditional methods of molecular pathology, this was a time consuming job that exploited precious tissue resources. However, a high throughput tissue analysis approach, [tissue microarray (TMA) technology], has been developed[64-66]. Using this TMA technology, samples from up to 1000 different tumors are arrayed in one recipient paraffin block, sections of which can be used for all kinds of *in situ* analyses[22,67].

Sections from TMA blocks can then be utilized for the simultaneous analysis of DNA, RNA or protein tumor levels. TMA protein analysis has also been performed in CRC samples for prognostic evaluation[68-72]. However, even though it has been suggested that minute arrayed tissue specimens are representative of their donor tissues, highly heterogeneous cancer types and low levels of protein expression could account for underestimating determined protein expression levels in certain tumors[68,73].

There are multiple different types of TMAs that can be utilized in cancer research including multi tumor arrays (containing different tumor types), tumor progression arrays (tumors of different stages) and prognostic arrays (tumors with clinical endpoints). The combination of multiple different TMAs allows a very quick but comprehensive characterization of biomarkers of interest.

Despite what proteomics have added to translational research in cancer, there are some novel approaches that combine the information provided by genomic and proteomic assays run in parallel in order to complement the translational impact of both procedures[74]. This has also been applied to CRC profiling. Kwong *et al*[75], for instance, studied gene and protein expression performed in parallel across progressive stages of human CRC. For this purpose, they applied cDNA microarray and 2D-PAGE technologies in parallel to analyzed samples collected from 60 CRC cases at various stages of disease progression. Of 47 genes analyzed, 12 (26%) showed significant correlation between mRNA level and protein levels, suggesting that protein abundance is regulated at the transcriptional level. The remaining 31 genes showed either a non-significant correlation between mRNA and protein expression levels or, in 28% of the genes, a negative correlation. Therefore, the authors conclude that posttranscriptional mechanisms play an important role in the regulation of gene products activities in CRC, underline the importance of analyzing gene expression at multiple levels and claim that genomic and proteomic approaches actually complement each other.

In another recent study to identify new biomarkers, Madoz-Gurpide *et al*[76] investigated the feasibility of expressing soluble proteins corresponding to up-regulated genes in surgically resected CRC samples. They used cDNA microarrays (CNIO Oncochip)[77] to identify differentially expressed genes in malignant compared to normal samples isolated from 22 different CRC patients. After investigating different sources of cDNA clones for protein expression, from 29 selected genes, 21 different proteins were finally expressed soluble with, at least, one distinct fusion protein. Additionally, seven of these potential markers were tested for antibody production and/or validation, confirming six of them to be overexpressed in CRC tissues by immunoblotting and TMA analysis[76]. Authors suggest that this kind of approach may provide relevant

biological information of the neoplastic processes and lead to a better characterization of potentially interesting markers in a quite straightforward way for early diagnosis or individualized prognosis assessment.

Oncogenic pathways: Validating target candidates

The previously reviewed development of genomics and proteomics in cancer research has yielded an uncountable number of new potential oncogenic mediators and checkpoints, in CRC, worth further investigating. These novel gene-depending elements, potential new targets for future drugs, are commonly involved in a variety of molecular pathways and their intimate upstream/downstream regulators as well as their crosstalk networks and functional relevance still need to be addressed.

Most widely used experimental methods for molecular pathway research in oncology are performed on fairly well-controlled *in vitro* systems. Recent cell biology achievements and discoveries however, have led to more reliable and physiologically relevant settings where observations on cell behavior and cell fate under particular conditions can be imported into *in vivo* experiments employing animal cancer models and even translating findings into new human therapeutic trials.

In the last few years, several approaches to find molecules able to inhibit the expression of genes (so-called gene-silencing molecules) involved in colorectal cancer progression and therapeutic resistance have been pursued. Sequence-specific gene suppression strategies using antisense oligonucleotides (AS-ODN), ribozymes and deoxyribozymes were initially described and developed[78-82]. AS-ODN derivatives, depending on their type, recruit RNase H to cleave the target mRNA or inhibit translation by steric hindrance. Ribozymes though, directly bind to RNA *via* Watson-Crick base pairing and cleave the phosphodiester backbone of the RNA target by transesterification. Similarly, deoxyribozymes also bind to their RNA substrates *via* Watson-Crick base pairing and specifically cleave the target RNA.

Currently, in addition to their value in target validation studies, different AS-ODN strategies are under evaluation in phase Ⅱ and Ⅲ clinical trials, particularly in hematological malignancies, malignant melanoma and prostate cancer[83,84]. However, consolidating AS-ODN as a broadly applicable functional genomic and therapeutic tool has proven difficult. For instance, difficulties in delivery of the AS-ODN into target tissues, instability of AS-ODN *in vivo*, poor oral availability, uncertainties about the precise mode of action, and toxic effects in animal and human studies have been argued[80,83]. Moreover, a number of class effects are observed with AS-ODN that are unrelated to the specific targeted mRNA sequence. Acute effects include activation of the alternative complement pathway and inhibition of the intrinsic coagulation pathway. In fact, given repeated doses of AS-ODN to animals, accumulation of AS-ODN and/or metabolites occurs in the form of basophilic granules in various tissues, including the kidney, lymph nodes and liver. Although several approaches are known to overcome some of these difficulties[85], very few contributions have firmly supported the use of AS-ODN technology in CRC research[86-88].

But in the field of gene-silencing molecules, the most recent and fascinating tools discovered for studying gene regulation and gene expression control are microRNAs (miRNAs) and small interfering RNA (siRNAs). miRNAs and siRNAs are typically 21 to 25 nucleotide RNA molecules that induce gene silencing by RNA interference (RNAi)[89-91]. Since the description of RNA interference (RNAi) in 1998[92], this gene-silencing technology has been developed into a widely used methodology in basic as well translational research. RNAi was originally discovered as a naturally occurring pathway in plants and invertebrates[92]. Once long double-stranded RNA molecules are inserted into these organisms, they are processed by the endonuclease Dicer into siRNAs. These siRNAs are subsequently incorporated into the multicomponent RNA-induced silencing complex (RISC), which unwinds the duplex and uses the anti-sense strand as a guide to look for homologous mRNAs and degrade them, as previously reviewed by others[93,94]. More strikingly, synthetic short siRNAs (20-25 bp) can be either delivered exogenously or expressed endogenously from RNA polymerase Ⅱ or Ⅲ promoters (in the form of siRNAs or short hairpin (sh)RNAs that are processed by Dicer into functional siRNAs) and used as a new powerful technology for achieving specific down-regulation of target mRNAs in mechanistic research or even therapeutic development in CRC[11,95-98].

Testing targeted therapies: Preclinical modeling in colorectal cancer

Once potential targets are discovered and their expression is successfully inhibited *in vitro*, the safety, efficacy and feasibility of their inhibitors need to be evaluated in animal models in which human disease can be faithfully reproduced. In fact, in the last years, the need of relevant *in vivo* models in colorectal cancer research has prompted many investigators to work on developing reliable, reproducible and human colorectal cancer-mimicking animal models[25,99,100].

However, in colorectal cancer, much has been learned from human inherited syndromes, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC)[101-103]. That knowledge in fact, has been translated into the design and development of CRC animal models.

Although several rat models have been created for the study of colorectal cancer[104-106], in this review, we will focus our attention on mouse models which have profusely evolved in the last few years because of their abundant genetic/genomic information, and easy mutagenesis using transgenic and gene knockout technology. Genetically engineered mice have become essential tools in both mechanistic studies and drug development in CRC, as previously reviewed by others[107]. In fact, mice provide unique opportunities to define and identify genes that are involved in colorectal cancer progression.

The first mouse model obtained to carry a mutation in the adenomatous polyposis coli (APC) tumor suppressor gene was named multiple intestinal neoplasia (Min)[108].

The Min mutation results in a truncated protein and induces the development of multiple intestinal adenomas (even more than one hundred) and a reduced lifespan of on average 150 d in heterozygous mice. Posterior models carrying mutations in different APC alleles have also been developed and each one possesses its own clinical manifestations. However, the majority of them shows small intestine adenomas and colonic tumors and distant metastases are rarely observed. Interestingly, it has been shown that different mutations in the APC gene, in *Apc1638N* mice for instance, confer distinct tumor susceptibility phenotypes and that fact resembles the heterogeneity observed in human FAP families[109]. Other models of hereditary non-polyposis colorectal cancer (HNPCC) have been developed through the mutation of several mismatch repair genes. One representative example are Msh2 deficient mice that are fertile and develop normally, however, these animals develop T-cell lymphomas early in their life and die because of the disease. Msh2 deficient mice that survive more than 6 months develop gastrointestinal adenomas, carcinomas and skin tumors and can also be used for tumorigenesis studies[110].

Finally, other more recent models have also been developed to better study colorectal cancer. Smad4 heterozygous mice bearing *Apc* mutations present an enhanced progression and a more malignant phenotype[111]. Other combinations responsible for increased gastrointestinal tumorigenesis are APC and oncogenic KRAS that seem to be synergistic in enhancing Wnt signaling[112].

CONCLUSIONS

Translational research is a key developing field in biomedicine. The direct application of basic research findings to the patient's diagnosis and treatment is even more important in cancer. In addition, clinical observations can dramatically contribute to basic research improvement and relevant enhancement. Colorectal cancer, due to its epidemiological importance and economic impact, is one of the main entities in which translational research is a reality today.

However, there still is a long way to go until basic researchers and clinical investigators share information and work together in colorectal cancer research on a daily basis.

Several new technologies and tools have demonstrated a great value in cancer and are in fact responsible for the last crucial pieces of research work allowing a new conception of cancer diagnosis and treatment. Among them, the development of new biomarkers for colorectal cancer combining proteomics and genomics is especially relevant.

Also, anti-sense strategies have recently opened the path for new target-specific therapy development. These new therapeutic discoveries need to be tested in preclinical animal models.

Since extensive validation of the above mentioned research fields is necessary, adequate funding is required. This may imply some adjustments in the current funding policy because it involves non-innovative studies. Furthermore, the pool of researchers/clinicians capable of performing translational research must be increased. Additionally, there should be an enhanced participation of patients in clinical trials and an optimization of the efficiency of these trials using validated surrogate markers. Only when these conditions are fulfilled the 'post-genomic' era of biomedical research will have unprecedented opportunities to innovate and improve therapy for cancer.

COMMENTS

Background

In the present paper, a summarized view of some of the new available approaches on colorectal cancer translational research is provided. Translational research in colorectal cancer comprises the identification and characterization of new molecular markers and the discovery of novel targeted therapies. The better understanding of human cancer and the design of more reliable tumor models and more accurate experimental systems is also part of translational research in cancer.

Research frontiers

The principal directions toward which translational research has spread and grown in colorectal cancer in recent years are genomics and proteomics, oncogenic pathways assessment and new targeted therapies discovery.

Innovations and breakthroughs

To our knowledge, there is no other published paper specifically focused on translational research in colorectal cancer. Therefore, we consider this review as a unique and inspiring one.

Applications

The main objective of this manuscript is to help scientists and physicians working on colorectal cancer determine which findings have been already achieved and which others are still underway and provide a better knowledge of new tools and techniques available for this purpose. This focus might inspire other authors in their own research projects and emphasize the need of a new approach to colorectal cancer research.

Terminology

Translational research: Investigation directed to the link of basic and clinical research in order to better define aims and better control tools and experimental systems. Genomics: Part of the bioscience that studies the genome and its implications in disease appearance, progression and response to treatment. Proteomics: Part of the bioscience responsible for peptide and protein investigation and their role in the diagnosis, treatment and research of disease. Targeted therapies: Group of drugs specifically designed to a certain target of the tumor cell such as growth factor receptors, membrane proteins and others.

Peer review

This manuscript is a very good and complete review of the topic exposed.

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Targeting hepatitis B virus antigens to dendritic cells by heat shock protein to improve DNA vaccine potency

Qin-Long Gu, Xue Huang, Wen-Hong Ren, Lei Shen, Bing-Ya Liu, Si-Yi Chen

Qin-Long Gu, Bing-Ya Liu, Department of Surgery, Shanghai Institute of Digestive Surgery, Affiliated Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
Xue Huang, Wen-Hong Ren, Lei Shen, Si-Yi Chen, Center for Cell and Gene Therapy, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, United States
Correspondence to: Dr. Qin-Long Gu, Department of Surgery, Shanghai Institute of Digestive Surgery, Affiliated Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, 227 Chongqing Nan Road, Shanghai 200025, China. qlgu@shsmu.edu.cn
Telephone: +86-21-63841391 Fax: +86-21-63842157
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Abstract

AIM: To investigate a novel DNA vaccination based upon expression of the HBV e antigen fused to a heat shock protein (HSP) as a strategy to enhance DNA vaccine potency.

METHODS: A pCMV-HBeAg-HSP DNA vaccine and a control DNA vaccine were generated. Mice were immunized with these different construct. Immune responses were measured 2 wk after a second immunization by a T cell response assay, CTL cytotoxicity assay, and an antibody assay in C57BL/6 and BALB/c mice. CT26-HBeAg tumor cell challenge test *in vivo* was performed in BALB/c mice to monitor anti-tumor immune responses.

RESULTS: In the mice immunized with pCMV-HBe-HSP DNA, superior CTL activity to target HBV-positive target cells was observed in comparison with mice immunized with pCMV-HBeAg (44% ± 5% *vs* 30% ± 6% in E: T > 50:1, $P < 0.05$). ELISPOT assays showed a stronger T-cell response from mice immunized with pCMV-HBe-HSP than that from pCMV-HBeAg immunized animals when stimulated either with MHC class Ⅰ or class Ⅱ epitopes derived from HBeAg (74% ± 9% *vs* 31% ± 6%, $P < 0.01$). ELISA assays revealed an enhanced HBeAg antibody response from mice immunized with pCMV-HBe-HSP than from those immunized with pCMV-HBeAg. The lowest tumor incidence and the slowest tumor growth were observed in mice immunized with pCMV-HBe-HSP when challenged with CT26-HBeAg.

CONCLUSION: The results of this study demonstrate a broad enhancement of antigen-specific $CD4^+$ helper, $CD8^+$ cytotoxic T-cell, and B-cell responses by a novel DNA vaccination strategy. They also proved a stronger antigen-specific immune memory, which may be superior to currently described HBV DNA vaccination strategies for the treatment of chronic HBV infection.

Key words: Hepatitis B virus antigen; Dendritic cell; Heat shock protein; DNA vaccine

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection continues to be a major human health problem, and there are about 350 million chronic HBV carriers worldwide[1]. Chronic HBV infection is associated with serious complications as a result of long-term sequelae such as liver cirrhosis or hepatocellular carcinoma[2]. The host immune response to HBcAg and HBeAg appears critical in both viral clearance and clinical resolution. The ultimate objective for rational vaccine design is the induction of pathogen immunity. In laboratory animals, DNA vaccine has proven to be a simple and effective method to generate protective immunity against a variety of pathogens, including HBV[3,4]. DNA vaccination that can induce both cellular and humoral immune responses has become an attractive immunization strategy against chronic HBV infection. Although it is known that DNA applied either i.m. or intradermally is primarily taken up by muscle cells or keratinocytes, it has become clear in recent years that professional APCs are essential for priming naive T cells following DNA injection. Accumulating evidence indicates that dendritic cells (DCs), the most potent APCs, play a critical role in the induction of immune responses by DNA vaccines[5-7]. Thus, enhancement of antigen presentation by DCs is an attractive strategy to increase the potency of DNA vaccines. However, a major problem of DNA vaccines is its limited potency, because only a very limited fraction of injected DNA molecules are taken up by DCs.

Recently, heat shock protein (HSP) was observed to elicit protective immunity to cancers and infectious

agents. The abilities of HSP include: (a) to chaperone peptides, including antigenic; (b) to interact with antigen presenting cells through a receptor; (c) to stimulate antigen presenting cells to secrete inflammatory cytokines; and (d) to mediate maturation of DCs, making them a one-stop shop for the immune system[8]. These properties also permit to use of HSP for developing a new vaccine. HSP has been reported to activate innate immune responses, to mediate the maturation of DCs, to upregulate proinflammatory cytokines[9-12], and to induce specific CTL responses[13,14].

In this study, we describe a novel DNA vaccination strategy to enhance uptake and presentation of antigens by DCs. Specifically, we developed a DNA vaccination based upon expression of the HBV e antigen fused to HSP, which are versatile immune regulators that chaperone antigenic peptides for MHC class Ⅰ and Ⅱ presentation by DCs. After vaccination, DNA is taken up by various cells that produce and secrete the antigen-HSP fusion proteins. The secreted fusion proteins, in addition to inducing B-cells, are efficiently captured and processed by DCs *via* receptor-mediated endocytosis, and then presented *via* MHC class Ⅰ and class Ⅱ molecules. This study demonstrates a broad enhancement of antigen-specific $CD4^+$ helper, $CD8^+$ cytotoxic T-cell, and B-cell responses by this DNA vaccination strategy, which may be superior to currently described HBV DNA vaccination approaches for the treatment of chronic HBV infection.

MATERIALS AND METHODS

Mice and cell lines

The mice used were female C57BL/6 and Balb/c mice, aged 4-5 wk. All mice were maintained in the animal facility at Baylor College of Medicine with approval of the Institutional Animal Care and Use Committee. The tumor cell lines EL-4, Trampc-2, and CT26 were purchased from the ATCC. EL-4 and Tampc-2 cells were cultured in DMEM medium and CT26 cells in RPMI 1640 medium both containing 10% heat-inactivated FBS (GIBCO) at 37℃ in an humidified 5% CO_2 atmosphere.

DNA constructs

The pCMV-HBeAg-HSP construct was generated by inserting HBeA (be derived from the precore open reading frame by cleavage of its C-terminus, nucleotide: 1901-2452 of HBV genome) & HSP-70 (StressGen Biotechnologies, Victoria, British Columbia, Canada) plasmid into a pCMV vector (Invitrogen, Carlsbad, CA,USA) with the cloning site HindIII & XbaI. Two control vectors, pCMV-HBeAg & pCMV-HSP, were also generated.

DNA preparation and immunization

Plasmid DNA was amplified in *Escherichia coli* DH5α and purified using an endotoxin-free purification kit (Qiagen) according to a standard protocol. Concentration was determined using the UV/Visible Spectrophotometer (Pharmacia Biotech) at 260 and 280 nm, and the material was adjusted to a final concentration of 1 mg/mL with endotoxin-free PBS (Sigma) and stored at -20℃. Mice were divided into 4 groups, which were immunized with different DNA vaccines including pCMV-HBeAg-HSP, pCMV-HBeAg, and pCMV-HSP. Controls were injected with PBS (C57BL/6 mice) or pCMV (Balb/c mice). Immunization method: mice were injected s.c. (C57BL/6) and i.m. (Balb/c) in quadriceps with 100 μg of DNA in 100 μL, two inoculations were carried out with an interval of 2 wk. Two weeks after the second immunization blood and spleens were collected, and BALB/c mice were challenged with CT26-HBeAg tumor cells.

Elispot for T-cell response assay

Elispot assays were used as a measure for T-cell response. Ninety-six well filtration plates (Milipore, Bedford, MA, USA) were coated with AN18 (anti-mouse IFN-γ, Mebtech) at the concentration of 10 μg/mL and kept at 4℃ overnight. Splenocytes were cultured in 96-well plates (1×10^6 cells/mL and 2×10^6 cells/mL) with RPMI 1640/10% FBS containing HEPES, 2MC, and NEAAS. Splenocytes from mice of different groups were stimulated with HBeAg class Ⅰ peptide (HBcAg93-100 peptide), HBeAg class Ⅱ peptide (HBcAg 120-131), or HBV protein (HBsAg, 227 amino acids, 24kD) (BD Pharmingen, SD, CA, USA) for comparing the effect of different DNA vaccinations on the T-cell response. The splenocytes derived from mice vaccinated with HBeAg-HSP were also stimulated with Trampc-2 class Ⅰ peptide (P117-139, WT1), CT26 class Ⅰ peptide (peptide AH1), Tyrosinase protein, Tyrosinase class Ⅰ peptide (Ty-4), Tyrosinase class Ⅱ peptide (Ty-5), and PMSA4 class Ⅱ peptide as controls. Proteins were added at a final concentration of 60 μg/mL, peptides at a final concentration of 30 μg/mL. All assays were performed in triplicates. After stimulation for 20 h at 37℃, the plate was washed with PBS and the second antibody (anti-mouse IFN-γ, Mebtech Mab R4-6A2 biotin) was added for a further incubation at 37℃ for 2 h. Avidin-HRP was added for 1 h at room temperature after washing, then 100 μL AEC was added to each well for coloring for 4 min after washing. The reaction was stopped by drying the membrane. The results were sent to Zellnet Consulting, Inc. (NY, USA) for test.

CTL cytotoxicity assays

CTL cytotoxic activity was determined using a ^{51}Cr-release assay. In brief, splenocytes obtained from mice 2 wk after the second immunization were cultured in 24 well plates with RPMI 1640/10% FBS containing HEPES, 2MC, NEAAS, and IL-2 (50 U/mL). Splenocytes were stimulated with HBeAg class Ⅰ peptide (HbcAg93-100 peptide) for 7 d, with changing half of the medium every 2 d. Target cell lines were cultured with IFN-γ (100 U/mL) for 24 h. EL-4 cells were pulsed with HBeAg class Ⅰ peptide and HBV protein (HBsAg, 227 amino acids, 24 kDa) as target cells. EL-4 cells pulsed with HBV non-related class Ⅰ peptide such as CT26 and Trampc-2 class Ⅰ peptides, non-pulsed EL-4 cells, and CT26 cells pulsed with HBeAg class Ⅰ peptide served as controls. All target and control cells were labeled with ^{51}Cr for 90 min. Cells were added to the wells at effector-to-target ratios ranging from 100:1 to 6.25:1 in triplicates. Plates were incubated at 37℃ for 5 h, before supernatants were collected and activity was assessed in a Gamma counter (Beckman, Fullerton, CA, USA).

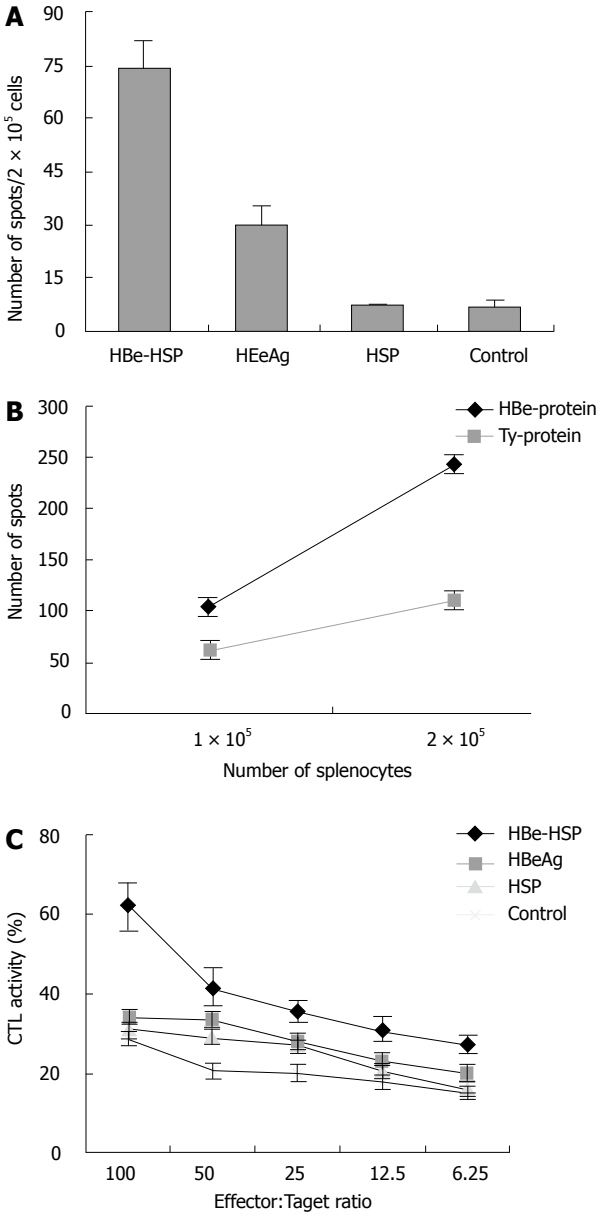

Figure 1 **A**: Comparison of T cell proliferation between different groups after stimulation with HBV protein (C57BL/6 mice); **B**: Comparison of T cell proliferation between stimulation with HBV protein and with non-related protein (Balb/c mice); **C**: Comparison of CTL activity to HBV protein pulsed target cells between different groups (C57BL/6 mice).

Anti-HBeAg antibodies assays

An ELISA assay (BD Biosciences) was used to quantify the antibody response after immunization. Sera were obtained 2 wk after the second immunization. Microtiter plates (Maxisorp) were coated with HBeAg overnight at 4℃. The coated plate was washed with PBS to stop reaction for 1 h at 37℃. Sera were added at different dilutions and the plate was incubated at 37℃ for 2 h. The second antibody was added at 37℃ for 2 h after washing with PBS. Finally, substrate was added and the plate was stored at room temperature for 30 min before stopping reactions with 4N sulfuric acid. Reading of the plate was done in an ELISA reader at 450 nm.

CT26-HBeAg tumor cell challenge test

CT26 cells were transfected with HBeAg using GenePoter reagent (Gene Therapy Systems) according to the manufacture's instructions. Forty-eight hours after transfection, cells were harvested and plated into selective medium in 10 cm dishes, 5×10^4 CT26 cells were plated into 250 μg/mL Geneticin. Two weeks after the second vaccination, 5×10^5 CT26 cells with HBeAg were injected s.c. into mice. Tumor incidence and tumor growth were monitored and tumor size was measured ($v = 1/2ab^2$: v: volume; a: largest diameter; b: smallest diameter).

Statistical analysis

All statistical analyses were performed using student *t*-test. Values of $P < 0.05$ was considered significant.

RESULTS

Enhancement of T cell response and CTL activity by HBeAg-HSP DNA vaccine

To evaluate whether HBeAg-HSP DNA vaccine can enhance immune response *in vivo*, splenocytes were obtained for T cell response and CTL activity. These immune responses were first stimulated with HBV protein and were compared between C57BL/6 and Balb/c mice immunized with different DNA vaccines. In CTL assay, EL-4 cells were pulsed with HBV protein as target cells. ELISPOT showed a stronger T-cell response from the mice immunized with HBeAg-HSP than that from HBeAg immunized mice after stimulation with HBV protein (Figure 1A, spots 74 ± 5 *vs* 31 ± 6, $P < 0.01$). A specific T-cell response was obtained in HBV protein stimulation in comparison with Tyrosinase protein stimulation (Figure 1B). Superior CTL assay to HBV protein pulsed target cell was also observed in mice immunized with HBeAg-HSP in comparison to those immunized with HBeAg (Figure 1C, 46% ± 10% *vs* 35% ± 8% in E: T > 50:1, $P < 0.05$).

We also evaluated the specific stimulating effect of HBV MHC class I peptide on T cell response and CTL activity. After splenocytes were stimulated with HBV MHC class I peptide, ELISPOT showed a stronger T-cell response from mice immunized with HBeAg-HSP than from those which had been immunized with HBeAg (Figure 2A, 76 ± 6 *vs* 29 ± 5, $P < 0.01$). CTL activity to HBV class I peptide pulsed target cells is also stronger in mice immunized with HBeAg-HSP than in mice immunized with HBeAg (Figure 2B, 44 ± 5 *vs* 30 ± 6 in E: T > 50:1, $P < 0.05$). A specific effect on T cell response is also obtained after stimulation with HBV class I peptide in comparison with CT26 class I peptide and TrampC-2 class I peptide stimulation (Figure 2C). A stronger CTL activity to HBV class I peptide pulsed target cells was shown in comparison with target cells pulsed with CT 26 class I peptide and TrampC-2 class I peptide (Figure 2D). T cell response to HBV class I peptide and CTL activity to HBV class I peptide pulsed target cells proved cytotoxic T cell activity.

To evaluate helper T cell activity, the effects of HBV MHC class Ⅱ peptides on T cell response and CTL activity were studied. Splenocytes were stimulated with HBV class Ⅱ peptides for T cell response. A stronger T cell response was obtained from HBeAg-

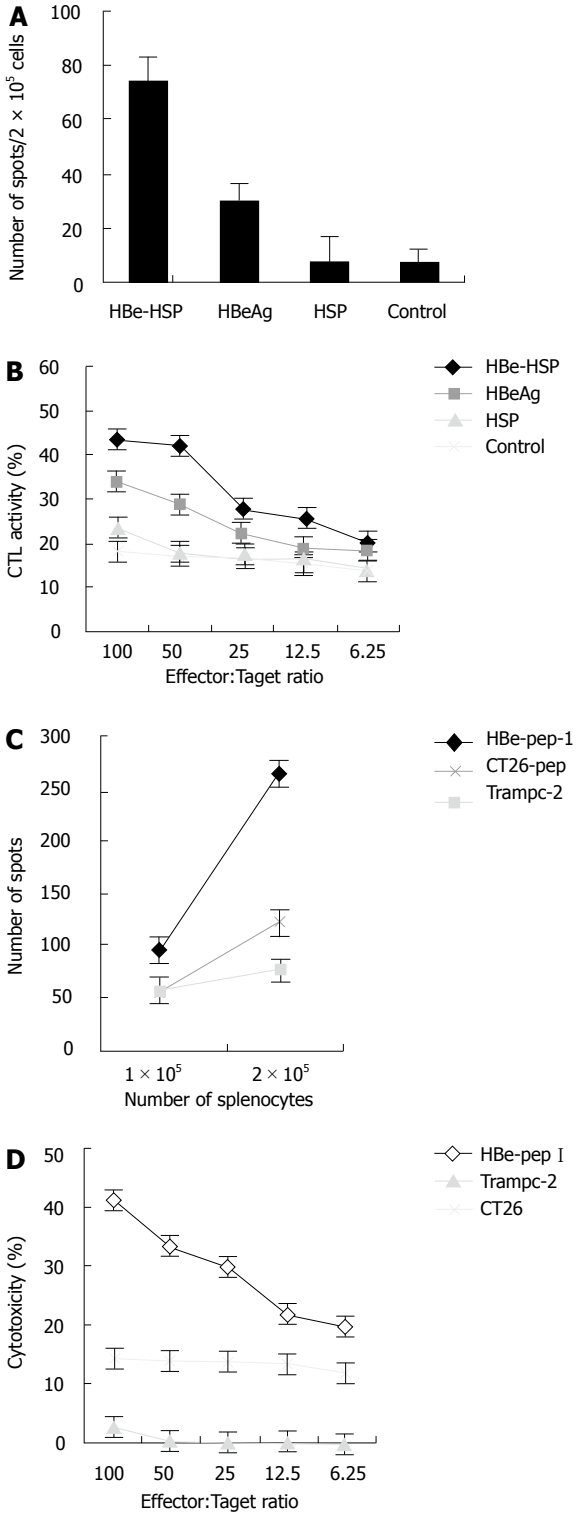

Figure 2 **A**: Comparison of T cell proliferation between different groups after stimulation with HBV class I peptide (C57BL/6 mice); **B**: Comparison of CTL activity to HBV class I peptide pulsed target cells between different groups (C57BL/6 mice); **C**: Comparison of T cell proliferation between stimulation with HBV and with non-related class I peptide (Balb/c mice); **D**: Comparison of CTL activity to HBV class I peptide pulsed target cell between HBV and non-related class I peptide (Balb/c mice).

HSP DNA vaccine immunized mice in comparison with that in mice immunized with HBeAg (A, spots 74 ± 9 *vs* 31 ± 6, $P < 0.01$) and HSP DNA vaccine (Figure 3). A specific stronger T cell response by HBV class Ⅱ peptide stimulation was shown in comparison with that by PSMA4 class Ⅱ peptide, Tyrosin-4 and Tyrosin-5 class Ⅱ peptide stimulation (Figure 3B).

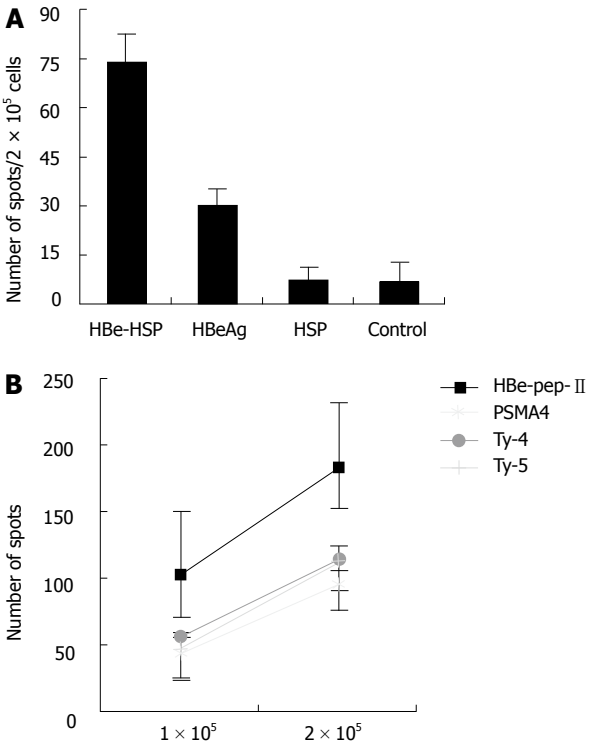

Figure 3 **A**: Comparison of T cell proliferation between different groups after stimulation with HBV class Ⅱ peptide (C57BL/6 mice); **B**: Comparison of T cell proliferation after stimulation with different class Ⅱ peptide (Balb/c mice).

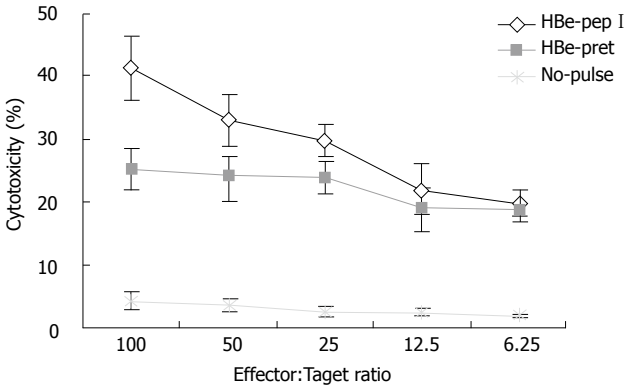

Figure 4 Comparison of CTL activity to target cells pulsed with different agents (Balb/c mice).

We also studied whether HBV related proteins and class I peptides can increase target cell antigenicity. Results suggested that splenocytes from mice immunized with HBeAg-HSP DNA vaccine have a stronger CTL activity to target cells pulsed with HBV protein and HBV class I peptide in comparison to non-target cells and CT 26 cells (Figure 4).

Serum antibody response to HBeAg antigen after DNA vaccination

To determine whether HBeAg-HSP DNA vaccination can also induce an antibody response to HBeAg antigen, we measured serum anti-HBeAg antibody responses by ELISA assay. As shown in Figure 5, antibody levels detected in mice immunized with the HBeAg-HSP DNA vaccine were markedly higher than those immunized with

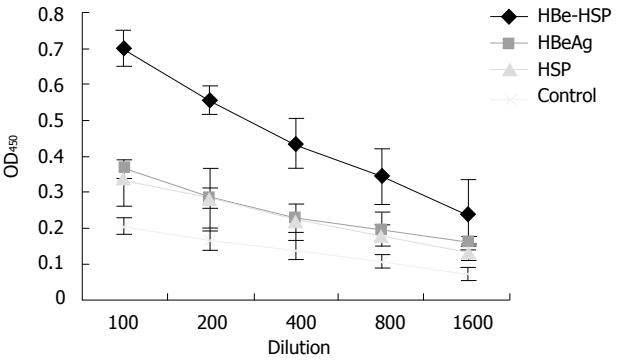

Figure 5 Comparison of antibody titers to HBV between different groups after immunization (Balb/c mice).

the HBeAg or the HSP DNA vaccines ($P < 0.05$). The results indicate the superiority of HBeAg-HSP in inducing humoral immunity.

Systemic immunity enhancement in vivo by HBeAg-HSP DNA vaccine

To evaluate systemic immunity enhancement *in vivo* by the HBeAg-HSP DNA vaccine, Balb/c mice (10 mice/group) were challenged by CT26 cells transfected with HBeAg to observe the antitumor effect after different DNA vaccine immunizations. CT26-HBeAg cells were injected s.c. at 5×10^5/mouse and tumor incidence and tumor growth were monitored. Results showed that there is a low tumor incidence in mice immunized with HBeAg DNA vaccine and a lower tumor incidence in HBeAg-HSP DNA vaccinated mice (Figure 6A), the incidence of tumor are 6/10 in HBeAg-HSP group, 8/10 in HBeAg group and 10/10 in the other two groups. Tumor growth was slowest in mice immunized with the HBeAg-HSP DNA vaccine (Figure 6B). The results suggested that HBeAg-HSP DNA vaccination can induce a stronger immune response to the related antigen.

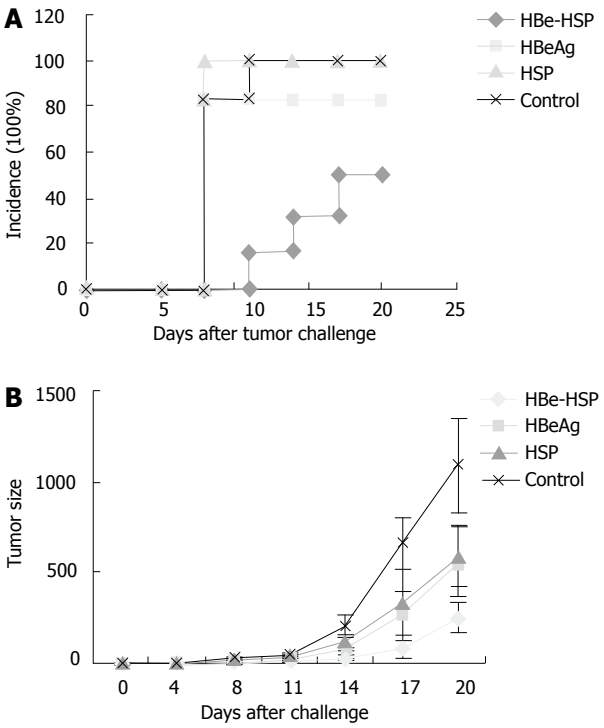

Figure 6 **A**: Tumor incidence after CT26-HBeAg challenge of Balb/c mice; **B**: Tumor growth after CT26-HBeAg challenge of Balb/c mice.

DISCUSSION

HBV infection is a major human health problem and it is associated with a risk of developing liver cirrhosis or hepatocellular carcinoma[1,2]. Thus, effective preventive and therapeutic strategy to chronic HBV infection has been a major exploration[15,16]. Only a small proportion of patients with chronic HBV infection benefit from a treatment with interferon-α (IFN-α)[2]. Antigen-based vaccines have some disadvantages, such as the possibility of reversion to a virulent form, especially in immunocompromised individuals; whole-killed or subunit vaccines do not induce intracellular synthesis of antigen because there is poor or absent presentation of antigen on class I MHC and thus poor induction of a CTL response[17]. DNA-based vaccination is an efficient new technique to stimulate specific immune responses and specific for HBV antigen to induce a strong humoral and cell-mediated immunity against HBV infection[18,19]. HBV(HBsAg, HBc/eAg) DNA vaccine has been popularly studied for prophylaxis or therapy against HBV infection[15,16,20-24].

DNA vaccine has made an attractive alternative to conventional methods of vaccination. HBV DNA vaccination can induce $CD8^+$ T cells as well as a dominant Th1 phenotype among the splenic lymphocytes, so eliciting strong CTL and protective levels of antibody[25-28]. Antigen-presenting cells (APCs) play a key role in induction of immune responses by DNA vaccines. DNA vaccines express native protein antigens in situ which can be recognized by B cells and presented by MHC class Ⅰ and Ⅱ molecules to prime helper T cells and CTLs. Dendritic cells (DCs) are usually thought of as a specific APCs for T cell and B cell activation and regulation of antibody synthesis, presentation of antigen by DCs is a potent stimulus to immune response, particularly to cell-mediated immunity and the development of CTLs. Thus, DCs are critical for initiating and modulating B and T cell responses elicited by DNA vaccination[29-31]. However, only a very limited fraction of injected DNA molecules is taken up by DCs, the intracellular antigens expressed by DCs are difficult to be processed and presented to MHC class Ⅱ[32].

In this study, we designed a novel DNA vaccination strategy to enhance uptake and presentation of antigen by DCs, specifically, we developed a DNA vaccine based upon the expression of the HBV e antigen fused to HSP, which are versatile immune regulators that chaperone peptides for MHC class I and Ⅱ presentation by DCs. The abilities of HSP include: to chaperone peptides, including antigen peptides; to interact with antigen presenting cells through a receptor; to stimulate antigen presenting cells, such as DCs to secrete inflammatory cytokines; and to mediate DC maturation[14]. The HSP70 peptide complex has been shown to elicit $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells and has been used for inducing antitumor immunity and for therapy of infectious diseases[33-37]. The novel vaccination strategy-HBeAg-HSP we developed

has been shown to induce a stronger CTL activity, T cell proliferative response, and antibody response than that of HBeAg DNA vaccine. Moreover, it also showed a stronger anti-tumor immunity to tumor with HBV antigen challenge than that of the HBeAg DNA vaccine. To date, many kinds of cancer vaccines have been tested worldwide and have shown their own advantages. HSP-based cancer vaccine is one of the outstanding representatives[38]. HSP complexes isolated from tumor have been shown to induce specific anti-tumor immunity, HSP alone can also induce non-specific immunity[39]. Recent works by Enomoto and Chan indicated HSP70 based vaccine possess superior properties such as stimulation of DC maturation and T cell proliferation[40,41]. HSP vaccine has been extensively tested in animals and more recently in clinical trials[42,43]. HSP vaccine can induce immune responses against mutated tumor-specific antigens, as well as normal self-antigens. Immune responses to self-antigens by HSP may thus produce damage to normal tissues, however, there are no reports about toxic side effects in mouse models or clinical trials with HSP[44,45].

It is important that exploit of effective DNA vaccination to induce HBV specific immune response to clear HBV infection. The results of this study demonstrate the broad enhancement of antigen-specific $CD4^+$ helper, $CD8^+$ cytotoxic T-cell, B-cell response, and specific anti-tumor immunity by this DNA vaccination strategy, which may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

COMMENTS

Background

Chronic HBV infection is associated with serious complications as a result of long-term sequelae such as liver cirrhosis or hepatocellular carcinoma. The host immune response to HBcAg and HBeAg appears critical in both viral clearance and clinical resolution. DNA vaccination that can induce both cellular and humoral immune responses has become an attractive immunization strategy against chronic HBV infection. However, a major problem of DNA vaccine is its limited potency, because only a very limited fraction of injected DNA molecules are taken up by DCs. In this study, we describe a novel DNA vaccination strategy to enhance uptake and presentation of antigens by DCs.

Research frontiers

A DNA vaccination based upon expression of the HBV e antigen fused to a heat shock protein (HSP) was developed, this study demonstrate that this DNA vaccination strategy may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

Innovations and breakthroughs

DNA vaccine has made an attractive alternative to conventional methods of vaccination. In this study, we designed a novel DNA vaccination strategy to enhance uptake and presentation of antigen by DCs, it may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

Applications

HSP vaccine has been extensively tested in animals and more recently in clinical trials. The results in this study suggested that HBeAg-HSP DNA vaccine may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

Terminology

DNA vaccine has made an attractive alternative to conventional methods of vaccination. HBV DNA vaccination can induce $CD8^+$ T cells as well as a dominant Th1 phenotype among the splenic lymphocytes, so elicit strong CTL and protective levels of antibody. The novel vaccination strategy-HBeAg-HSP we developed has been shown to induce a stronger CTL activity, T cell proliferative response, and antibody response than the HBeAg DNA vaccine, and it also showed a stronger anti-tumor immunity to tumor with HBV antigen challenge than that of HBeAg DNA vaccine.

Peer review

The novel vaccination strategy-HBeAg-HSP was studied and it is one of the outstanding representatives for the treatment of chronic HBV infection. Moreover, it will be interesting in the treatment of cancer in future. It is deserved to be published.

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CLINICAL RESEARCH

Stability of cirrhotic systemic hemodynamics ensures sufficient splanchnic blood flow after living-donor liver transplantation in adult recipients with liver cirrhosis

Tomohide Hori, Shintaro Yagi, Taku Iida, Kentaro Taniguchi, Kentaro Yamagiwa, Chiduru Yamamoto, Takashi Hasegawa, Koichiro Yamakado, Takuma Kato, Kanako Saito, Linan Wang, Mie Torii, Yukinobu Hori, Kan Takeda, Kazuo Maruyama, Shinji Uemoto

Tomohide Hori, Kentaro Taniguchi, Kentaro Yamagiwa, Chiduru Yamamoto, Departments of Hepatobiliary Pancreatic Surgery, Mie University Hospital, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Takuma Kato, Linan Wang, Mie Torii, Department of Cellular and Molecular Immunology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Shintaro Yagi, Taku Iida, Shinji Uemoto, Department of Hepatobiliary Pancreatic and Transplant Surgery, Kyoto University Hospital, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan
Takashi Hasegawa, Kazuo Maruyama, Department of Anesthesiology and Critical Care Medicine, Mie University Hospital, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Koichiro Yamakado, Kan Takeda, Department of Radiology, Mie University Hospital, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Kanako Saito, Department of Medical Oncology and Immunology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Yukinobu Hori, Nagoya Economic University Graduate School of Law, 61-1 Uchikubo, Inuyama City, Aichi Prefecture, 484-8504, Japan
Correspondence to: Tomohide Hori, Department of Hepatobiliary Pancreatic Surgery, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan. tomohide@clin.medic.mie-u.ac.jp
Telephone: +81-59-2321111 Fax: +81-59-2328095
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Abstract

AIM: To investigate the correlation between systemic hemodynamics and splanchnic circulation in recipients with cirrhosis undergoing living-donor liver transplantation (LDLT), and to clarify how systemic hemodynamics impact on local graft circulation after LDLT.

METHODS: Systemic hemodynamics, indocyanine green (ICG) elimination rate (K_{ICG}) and splanchnic circulation were simultaneously and non-invasively investigated by pulse dye densitometry (PDD) and ultrasound. Accurate estimators of optimal systemic hyperdynamics after LDLT [i.e., balance of cardiac output (CO) to blood volume (BV) and mean transit time (MTT), defined as the time required for half the administered ICG to pass through an attached PDD sensor in the first circulation] were also measured. Thirty recipients with cirrhosis were divided into two groups based on clinical outcomes corresponding to postoperative graft function.

RESULTS: Cirrhotic systemic hyperdynamics characterized by high CO, expanded BV and low total peripheral resistance (TPR) were observed before LDLT. TPR reflecting cirrhotic vascular alterations was slowly restored after LDLT in both groups. Although no significant temporal differences in TPR were detected between the two groups, CO/BV and MTT differed significantly. Recipients with good outcomes showed persistent cirrhotic systemic hyperdynamics after LDLT, whereas recipients with poor outcomes presented with unstable cirrhotic systemic hyperdynamics and severely decreased K_{ICG}. Systemic hyperdynamic disorders after LDLT impacted on portal venous flow but not hepatic arterial flow.

CONCLUSION: We conclude that subtle systemic hyperdynamics disorders impact on splanchnic circulation, and that an imbalance between CO and BV decreases portal venous flow, which results in critical outcomes.

Key words: Cirrhosis; Hyperdynamic; Portal hypertension; Splanchnic; Indocyanine green

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INTRODUCTION

We previously demonstrated that systemic hemodynamics

affecting postoperative graft function are crucial for living-donor liver transplantation (LDLT)[1]. However, the relationship between systemic hemodynamic parameters and splanchnic circulation after LDLT remains to be fully elucidated. In particular, the influence of the systemic hemodynamic state on splanchnic circulation is unclear. Therefore, we carried out a detailed investigation of systemic and splanchnic hemodynamic behavior after LDLT in adult recipients with cirrhosis.

Prior to undergoing LDLT, recipients with cirrhosis generally develop peculiar systemic and splanchnic hemodynamics due to portal hypertension[2-4]. To ascertain correlations between systemic hemodynamics and splanchnic circulation, and to clarify how the systemic hemodynamic state impacts on the local graft circulation, we performed simultaneous assessments of systemic hemodynamics and directly measured splanchnic circulation by systemic dye distribution and ultrasound. We also determined the hemodynamic state required for an excellent clinical outcome corresponding to good graft function.

MATERIALS AND METHODS

Patients

From June 2003 to March 2006, indocyanine green (ICG) pharmacokinetics were analyzed using a non-invasive method in 30 adult recipients (average age 53.1 ± 9.3 years; 25 males, five females) who underwent orthotopic LDLT at Mie University Hospital. As well, splanchnic circulatory parameters were simultaneously assessed using Doppler ultrasound. All 30 patients received a right-lobe liver graft. Clinical diagnoses were 26 cases of liver cirrhosis with hepatitis B or C (18 complicated by hepatocellular carcinoma), two cases of biliary atresia (result of postoperative state of Kasai's operation at childhood), and one case each of primary sclerosing cholangitis and alcoholic liver cirrhosis. All recipients were diagnosed with liver cirrhosis, based on histopathological examination of resected specimens. ABO blood group compatibility was identical in 24 recipients and compatible in six. The operative procedures and immunosuppression protocols used in our institute have been described in detail elsewhere[1,5-8]. All the protocols used in the present study were approved by the Ethics Review Committee for Human Studies of Mie University Graduate School of Medicine (Tsu, Mie, Japan), based on the Ethical Guidelines of the Helsinki Declaration of 1975. Informed consent was obtained from all patients before enrollment.

ICG, pulse dye densitometry (PDD) and analytical procedures

ICG is widely used for analysis of liver function[9,10]. Furthermore, the dye dilution curve of ICG can be used for measuring hemodynamic parameters[9,11]. A non-invasive method for measuring systemic hemodynamic parameters using ICG has been reported[12] and is relatively reliable compared with invasive ones[11,13-15]. It is also advantageous for clinical use because it is simple to use at the bedside, has quick real-time presentation of results and is cost-effective[16,17]. Hence, we used this non-invasive method in the present study.

ICG (Diagnogreen Inj., Daiichi Pharmaceutical, Tokyo, Japan), a non-toxic dye, has no known side effects other than a rare iodine allergy. Although a total of 630 ICG bolus injections were performed in the 30 recipients, no allergic responses or any other side effects were observed.

PDD, which measures the absorption of hemoglobin and ICG, is based on the principle of pulse spectrophotometry; the basic principles of which has been detailed elsewhere[11,12]. A PDD apparatus (DDG-2001; Nihon Kohden, Tokyo, Japan) was used to measure blood ICG concentrations and analyze dye densitography. A sensor was placed on the nose of each patient before ICG injection.

Twenty milligrams of ICG was injected through a peripheral cannula and immediately flushed with 20 mL normal saline[1,9,18]. PPD measurements were obtained before LDLT and from 1 to 14 d and at 21 d and 28 d postoperatively. In particular, measurements were performed every 12 h until 72 h postoperatively, because the hemodynamic parameters showed marked changes during the early postoperative period.

Systemic hemodynamic parameters and ICG elimination rate

The following parameters were measured and calculated using the PDD apparatus with the patients in a settled recumbent position: cardiac output (CO, L/min), cardiac index (CI, L/min per m^2), mean transit time (MTT, s, blood volume (BV, L), heart rate (HR, beats/min) and ICG elimination rate constant (K_{ICG}). MTT was defined as the time required for half the administered ICG to pass through the attached nasal sensor in the first circulation. Details of the above calculations have been described elsewhere[11,12,17]. Measurement of mean arterial pressure (MAP) was performed simultaneously with the PDD. MAP, calculated as MAP (mmHg) = (pulse pressure/3) + diastolic pressure, was measured using a standard manual method[19]. Total peripheral resistance (TPR) was subsequently calculated according to the following formula: TPR (dyne/s^5 per cm) = MAP × 80/CO[19].

Doppler ultrasound and splanchnic hemodynamic measurements

Doppler ultrasound assessment of splanchnic hemodynamic parameters was conducted at the same time as PDD. Portal venous flow velocity (PVFVe), portal venous flow volume (PVFVo), hepatic arterial pulsatility index (HAPI), and hepatic arterial resistance index (HARI) were evaluated as splanchnic circulatory parameters. A Triplex Doppler ultrasound system (Prosound SSD-5000SV; ALOKA, Tokyo, Japan) and a convex probe (2-5 MHz; UST-9119; ALOKA) were used for the Doppler ultrasound assessment. The following parameters were measured at the extrahepatic but post-anastomosis area: (1) PVFVe (cm/s), representing the mean of the maximal flow velocity of the portal vein; (2) PVFVo (mL/min), calculated from a cross-sectional area, assuming a circular portal vein section, and the mean velocity; (3) HAPI,

Table 1 Systemic hemodynamic parameters, K_{ICG} values and splanchnic circulatory parameters before LDLT

Parameters	Healthy individuals $n = 16$	Group Ⅰ $n = 25$	Group Ⅱ $n = 5$
Systemic hemodynamics			
CO (L/min)	5.83 ± 1.52	6.87 ± 0.97[a]	7.36 ± 1.07[e]
CI (L/min per m^2)	3.22 ± 0.71	4.10 ± 0.71[b]	4.56 ± 0.58[e]
BV (L)	3.40 ± 0.96	4.09 ± 0.51[a]	4.40 ± 0.45[e]
CO/BV (/min)	1.74 ± 0.28	1.69 ± 0.21	1.69 ± 0.28
MTT (s)	16.1 ± 2.3	16.5 ± 1.5	16.5 ± 1.2
HR (beat/min)	64.3 ± 9.9	77.9 ± 12.6[b]	77.6 ± 9.8[e]
MAP (mmHg)	89.3 ± 11.8	68.9 ± 6.5[d]	70.8 ± 11.2[e]
TPR (dyne/s^5 per cm)	1275.1 ± 228.3	818.9 ± 166.7[d]	785.3 ± 187.4[e]
ICG clearance test			
K_{ICG}	0.227 ± 0.076	0.037 ± 0.017[d]	0.056 ± 0.038[e]
Splanchnic circulation			
Portal vein			
PVFVo (mL/min)	1482.1 ± 335.6	327.3 ± 416.9[d]	435.6 ± 592.6[e]
PVFVe (cm/s)	45.1 ± 8.1	7.9 ± 12.8[d]	10.5 ± 13.8[f]
Hepatic artery			
HAPI	0.95 ± 0.11	1.06 ± 0.28[a]	1.16 ± 0.21[e]
HARI	0.93 ± 0.26	1.04 ± 0.23[a]	1.10 ± 0.10[e]

There were no significant differences between Groups I and Ⅱ in each parameter, respectively ($P > 0.05$, analyzed by Mann-Whitney's U test). Statistical differences between healthy individuals and Group I analyzed by Mann-Whitney's U test ([a]$P < 0.05$, [b]$P < 0.005$, [d]$P < 0.0005$). Statistical differences between healthy individuals and Group Ⅱ analyzed by Mann-Whitney's U test ([e]$P < 0.05$, [f]$P < 0.005$). ICG: Indocyanine green; LDLT: Living-donor liver transplantation; CO: Cardiac output; CI: Cardiac index; BV: Blood volume; MTT: Mean transit time; HR: Heart rate; MAP: Mean arterial pressure; TPR: Total peripheral resistance; PVFVo: Portal veinous flow volume; PVFVe: Portal venous flow velocity; HAPI: Hepatic arterial pulsatility index; HARI: Hepatic arterial resistant index.

calculated from the Doppler trace over one cardiac cycle as: (peak systolic velocity-minimum velocity)/mean of maximal velocities; and (4) HARI, derived from the Doppler spectrum over one cardiac cycle according to: (peak systolic velocity-end diastolic velocity)/peak systolic velocity. The measurement methods for the above indices have been described in detail elsewhere[20-23].

Establishment of normal ranges of systemic hemodynamic parameters, K_{ICG} value and splanchnic circulatory parameters

To establish the normal ranges of the variables we investigated the variables using the above-described methods in seven donors before LDLT and in nine volunteers who agreed to the aims of this study. The data measured in these 16 healthy individuals represent the normal ranges of the parameters, and are shown in Table 1. The control population showed no significant differences in age or body surface area compared with the LDLT recipients (data not shown).

Computed tomographic (CT) volumetry of liver grafts and the standard liver volume (SLV)

In our institution, helical CT studies are routinely performed at 2 and 4 wk after LDLT. All 30 recipients underwent these studies after LDLT. The helical CT studies were conducted using a High Speed Advantage QX-1 (GE Medical Systems, Tokyo, Japan). The scanning parameters were 120 kV, 200 mA, collimation of 5 mm, and a table speed of 15 mm/rotation, with reconstruction increments of 5 mm. Graft volume was calculated by CT volumetry. SLV was calculated according to a previously described formula[24].

Technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl-human serum albumin (99mTc-GSA) liver scintigraphy and ratio of liver to heart-plus-liver radioactivity at 15 min (LHL15)

Since asialoglycoprotein receptors on hepatocytes are characteristic of functional liver cells[25], 99mTc-GSA liver scintigraphy is used as a reliable assessment tool for functional hepatic volume[26]. A total of 60 measurements were performed in the 30 recipients at 2 and 4 wk after LDLT. After intravenous injection of 185 MBq of 99mTc-GSA (Nihon Medi-Physics, Nishinomiya, Japan), dynamic imaging was performed with the patient in the supine position using a large field-of-view gamma camera (GCA7200A; Toshiba, Tokyo, Japan). LHL15 was calculated by dividing the radioactivity of whole liver regions of interest (ROIs) by that of whole liver-plus-heart ROIs at 15 min after injection, as previously described[27].

Histopathological analysis and graft parenchymal damage score

In our institution, needle biopsies are performed after LDLT if necessary. Protocol biopsies are not performed because of the associated risks, such as hemorrhage[28]. In the present study, a total of 30 biopsy specimens from the 30 recipients were assessed within 4 wk after LDLT.

Tissue specimens were stained with hematoxylin-eosin using standard histopathological techniques, and reviewed by an experienced liver pathologist using a semi-quantitative scoring system for features of the graft parenchyma. The graft parenchymal damage score, representing liver damage, was calculated as the total of the following parenchymal feature scores: hepatocyte ballooning (0, no; 1, yes), hepatocyte necrosis (0, none; 1, small foci; 2, confluent areas; 3, bridging necrosis), congestion (0, no; 1, yes), the fraction of hepatocytes that contain microvesicular fat (0, none; 1, < 1/3 of hepatocytes, 2, between 1/3 and 2/3 of hepatocytes; 3, > 2/3 of hepatocytes), neutrophil aggregates (0, none; 1, minimal; 2, moderate; 3, extensive) and cholestasis (0, none; 1, mild; 2, moderate; 3, severe). The graft parenchymal damage score, which was modified from the score according to Neil *et al*[29], has been described in detail elsewhere[6].

Outcomes after LDLT

The clinical courses of all recipients were followed for 996.2 ± 436.5 d, ranging from 32 (patient died) to 1472 d after LDLT. The 30 recipients were retrospectively divided into two groups based on clinical outcomes corresponding to postoperative graft function. Although 25 recipients (Group Ⅰ) presented with a good clinical course and excellent outcome, a subset of five recipients (Group Ⅱ) required long-term intensive management, and finally died because of hepatic or extrahepatic reasons that

Table 2 Clinical profiles before, during and after LDLT

Clinical profile	Group Ⅰ (n = 25)	Group Ⅱ (n = 5)	P value[a]
Before LDLT			
Age	51.8 ± 9.8	58.6 ± 3.5	NS
Body surface area (m^2)	1.69 ± 0.18	1.61 ± 0.12	NS
Child-Pugh score (points)	9.2 ± 2.3	10.8 ± 2.2	NS
Model for end-stage liver disease score (points)	17.6 ± 6.7	17.4 ± 7.1	NS
During LDLT			
Native liver weight (g)	857.0 ± 227.5	946.0 ± 376.2	NS
Portal venous pressure before removal of native liver (mmHg)	21.5 ± 4.7	24.6 ± 7.1	NS
Cold ischemic time (min)	163.7 ± 79.0	139.6 ± 52.6	NS
Warm ischemic time (min)	55.1 ± 16.7	45.8 ± 12.7	NS
Anhepatic phase (min)	209.2 ± 104.9	184.4 ± 177.6	NS
Operative time (min)	899.4 ± 126.7	933.4 ± 131.0	NS
Blood loss (mL)	22515.7 ± 14200.5	22788.6 ± 19247.8	NS
Graft weight (g)	687.8 ± 124.6	632.0 ± 72.9	NS
Graft-recipient weight ratio	1.09 ± 0.21	1.23 ± 0.37	NS
After LDLT			
Intensive care unit stay (d)	5.1 ± 1.9	35.6 ± 15.7	< 0.005
%SLV based on CT volumetry			
2 wk after LDLT	1.14 ± 0.22	1.07 ± 0.09	NS
4 wk after LDLT	1.05 ± 0.15	1.17 ± 0.16	NS
LHL15 based on ^{99m}Tc-GSA liver scintigraphy			
2 wk after LDLT	0.935 ± 0.026	0.846 ± 0.061	< 0.005
4 wk after LDLT	0.941 ± 0.017	0.751 ± 0.034	< 0.005
Histopathological fraft parenchymal damage score (points)			
Within 4 wk after LDLT	3.9 ± 1.4	10.6 ± 1.3	< 0.005

Statistical differences between Groups I and Ⅱ analyzed by Mann-Whitney's U test (NS: $P > 0.05$). LDLT: Living-donor liver transplantation; SLV: Standard liver volume; CT: Computed tomographic; LHL15: The ratio of liver to heart-plus-liver radioactivity at 15 min; 99mTc-GSA: Technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl-human serum albumin.

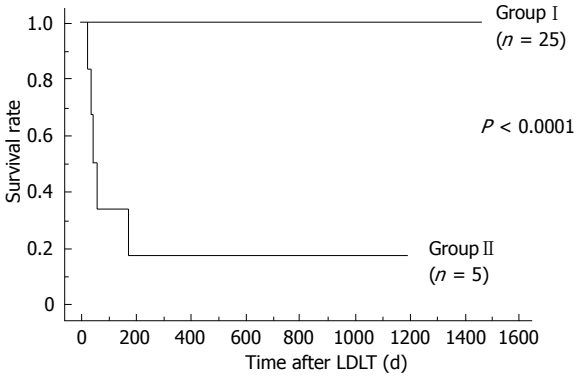

Figure 1 Survival rates after LDLT. The two lines represent the survival rates for Groups Ⅰ and Ⅱ. The P value analyzed by the log-rank test was < 0.0001.

originated from graft dysfunction with prolonged jaundice. Group Ⅱ showed poor clinical outcome, and survival rate differed significantly between the two groups ($P < 0.0001$) (Figure 1).

Clinical profiles before, during and after LDLT

There were no significant differences in the clinical profiles before and during LDLT between the two groups. We considered that high portal venous pressure before removal of the native liver was due to portal hypertension. After LDLT, there was a significant difference in the length of stay in the intensive care unit between the two groups. Although there were no significant differences in SLV, LHL15 and graft parenchymal damage scores both differed significantly between the two groups (Table 2). Because LHL15 and graft parenchymal damage scores accurately reflect functional hepatocytes, these results clearly indicated graft dysfunction in Group Ⅱ during the late postoperative period after LDLT.

Statistical analysis

Results were expressed as means ± SD. For individually, temporally and repeatedly measured data, differences in the changes over time after LDLT between the two groups were analyzed by repeated-measures ANOVA[30,31]. Differences in unpaired discontinuous data between the two groups were analyzed by Mann-Whitney's U test. Survival rates were calculated by the Kaplan-Meier method, and the log-rank test was used for between-group comparisons of recipient survival. All calculations were performed using Stat View-J 5.0 statistical software (SAS Institute, Cary, NC, USA) and values of $P < 0.05$ were considered significant.

RESULTS

Systemic hemodynamic states before LDLT and temporal differences in systemic hemodynamic parameters after LDLT

Cirrhotic systemic hemodynamics have been symbolized as hyperdynamic[1-4,32], and the hyperdynamic state characterized by high CO or CI, large BV, low TPR, mild tachycardia, and low or normal MAP[2,19,32-35]. Although hyperdynamic states were recognized in both groups, there were no significant differences between the two groups before LDLT. Interestingly, CO/BV and MTT were both constant before LDLT (Table 1). There were significant

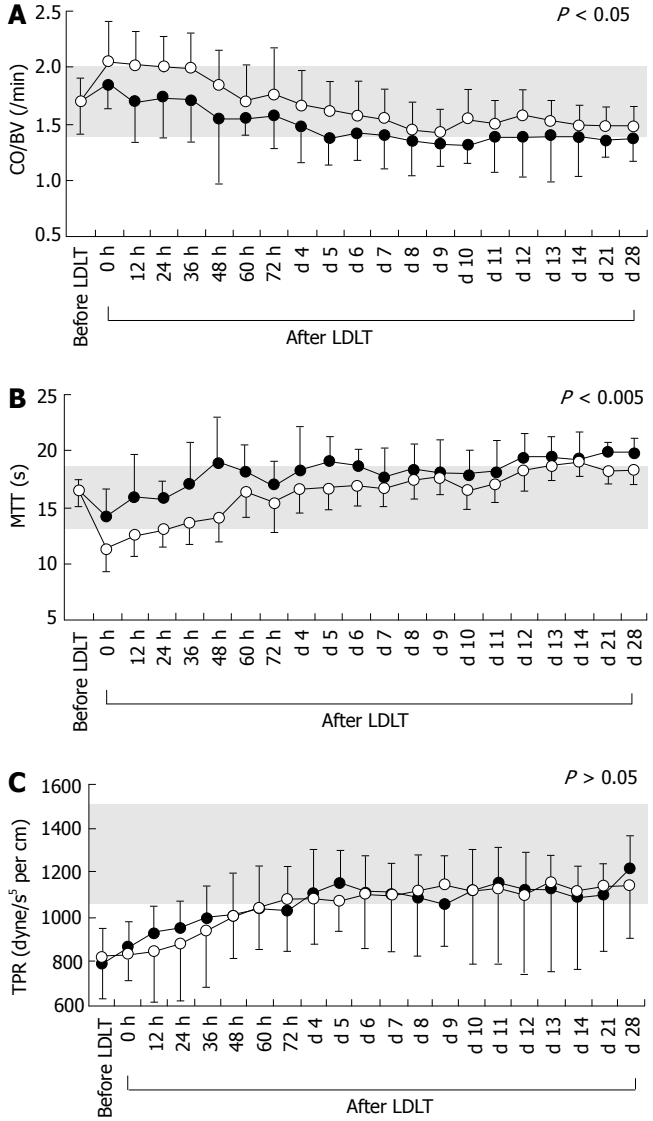

Figure 2 Temporal changes in systemic hemodynamic parameters before and after LDLT. **A:** Temporal changes in the ratio of CO to BV before and after LDLT; **B**: Temporal changes in MTT before and after LDLT; **C**: Temporal changes in TPR before and after LDLT. Open and closed circles represent systemic hemodynamic parameters for Groups Ⅰ and Ⅱ, respectively. Shaded areas show normal ranges measured in healthy individuals.

temporal differences after LDLT between the groups for CO/BV and MTT, but no significant differences in CO, CI, BV, HR, MAP or TPR (Table 3). The actual temporal changes in CO/BV, MTT and TPR are presented in Figure 2. When the absolute values of CO and BV in the recipients were compared with those of healthy individuals, recipients in Group Ⅰ persisted in a hyperdynamic state after LDLT, while those in Group Ⅱ showed a tendency to remain in a hyperdynamic state (actual temporal changes not shown). Thus, regardless of the outcome and graft function, the temporal changes in the absolute values of CO and BV between groups did not reach statistical significance. Therefore, as we have previously determined, detecting subtle disorders of optimal systemic hemodynamics in recipients with cirrhosis by comparing absolute values is not necessarily satisfactory (unpublished data). Indicators for peripheral resistance are thought to precisely reflect cirrhotic vascular alterations and the

Table 3 Statistical differences in post-operative temporal changes of systemic hemodynamic parameters, K_{ICG} values and splanchnic circulatory parameters

Parameters	Statistical temporal differences after LDLT between Groups Ⅰ and Ⅱ *P* value[1]
Systemic hemodynamics	
CO (L/min)	0.2321
CI (L/min per m^2)	0.5037
BV (L)	0.3420
CO/BV (/min)	0.0426[a]
MTT (s)	0.0023[b]
HR (beat/min)	0.0701
MAP (mmHg)	0.2453
TPR (dyne/s^5 per cm)	0.8859
ICG clearance test	
K_{ICG}	0.0001[d]
Splanchnic circulation	
Portal vein	
PVFVo (mL/min)	0.0113[a]
PVFVe (cm/s)	0.0171[a]
Hepatic artery	
HAPI	0.2504
HARI	0.4261

[1]Statistical temporal differences between Groups I and Ⅱ analyzed by repeated measures ANOVA ([a]$P < 0.05$, [b]$P < 0.005$, [d]$P < 0.0005$). ICG: Indocyanine green; LDLT: Living-donor liver transplantation; CO: Cardiac output; CI: Cardiac index; BV: Blood volume; MTT: Mean transit time; HR: Heart rate; MAP: Mean arterial pressure; TPR: Total peripheral resistance; PVFVo: Portal veinous flow volume; PVFVe: Portal venous flow velocity; HAPI: Hepatic arterial pulsatility index; HARI: Hepatic arterial resistant index.

presence of collateral vessels and shunts[19,36,37]. It should be noted that the changes in TPR in the two groups exhibited similar patterns with no prompt restoration, despite normalization of the portal pressure after LDLT, and showed quite slow improvement (Figure 2C).

K_{ICG} before LDLT and differences in temporal changes in K_{ICG} after LDLT

Recipients in both groups showed large decreases in K_{ICG} before LDLT (Table 1). Although there were no significant differences in K_{ICG} between the groups before LDLT, K_{ICG} changed significantly after LDLT (Figure 3, Table 3). The K_{ICG} value is dualistic, since it reflects functional hepatocytes and splanchnic blood flow[9,38-40]. However, splanchnic blood flow is a major determinant of K_{ICG} in normal liver[9,41,42]. We have previously demonstrated that K_{ICG} accurately evaluates functional hepatocytes during the late postoperative period, and sharply reflects splanchnic circulation during the early postoperative period, since LDLT restores functional hepatocyte volume drastically and immediately[1]. Extraordinary decreases in K_{ICG} from the early postoperative period were observed in Group Ⅱ, in contrast to the findings for Group I. Therefore, in the present study we verified the detailed splanchnic circulatory parameters measured by Doppler ultrasound.

Splanchnic hemodynamics before LDLT and temporal differences in splanchnic circulatory parameters after LDLT

Cirrhotic splanchnic circulation is symbolized by decreased

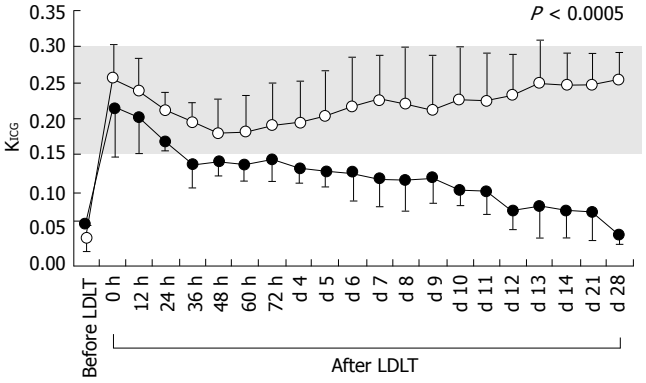

Figure 3 Temporal changes in K_{ICG} before and after LDLT. Open and closed circles represent K_{ICG} values for Groups Ⅰ and Ⅱ, respectively. The shaded area shows the normal range measured in healthy individuals.

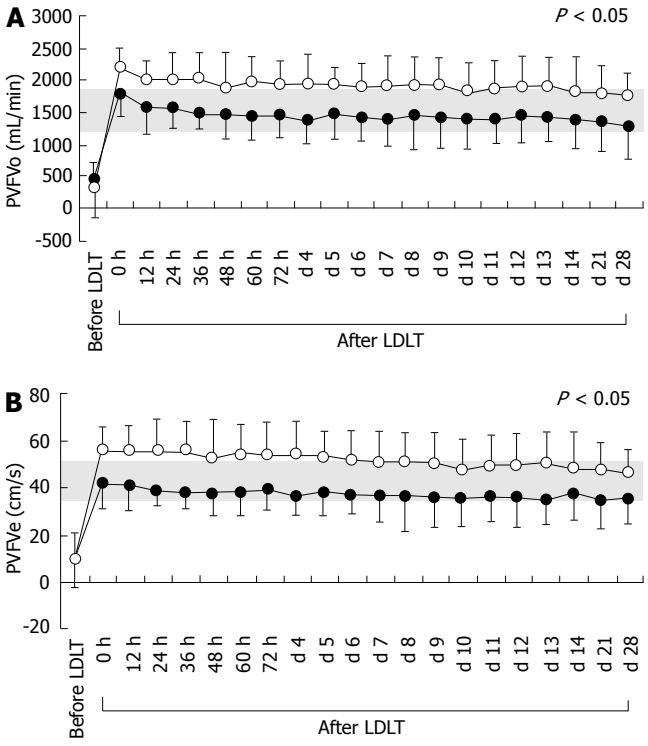

Figure 4 Temporal changes in splanchnic circulatory parameters before and after LDLT. **A:** Temporal changes in PVFVo before and after LDLT; **B**: Temporal changes in PVFVe before and after LDLT. Open and closed circles represent splanchnic circulatory parameters for Groups Ⅰ and Ⅱ, respectively. Shaded areas show normal ranges measured in healthy individuals.

portal venous flow because of portal hypertension, despite a systemic hyperdynamic state. Although all splanchnic circulatory parameters in both groups before LDLT differed significantly from those in healthy individuals, there were no significant differences between the two groups (Table 1). However, after LDLT, there were significant temporal differences in PVFVo and PVFVe, but no significant differences in HAPI and HARI, between the two groups (Table 3). The actual temporal changes in PVFVo and PVFVe are shown in Figure 4. Interestingly, differences in portal venous parameters, but not hepatic arterial parameters, were observed.

DISCUSSION

Almost all adult recipients who undergo LDLT develop liver cirrhosis with long-term portal hypertension. Portal hypertension results in vascular dilatation and collateral pathways. Thus, various alterations in systemic hemodynamics and splanchnic circulation occur, and adult recipients often present characteristic hemodynamics before LDLT. Cirrhotic hemodynamic abnormalities were obviously present before LDLT in the present study.

Several investigators have demonstrated that the systemic hyperdynamic state remains despite normalization of liver function and restoration of portal pressure after LDLT[19,33,36,43-45], and have suggested that most systemic parameters are slowly restored to the normal range after LDLT[19,36]. In agreement with these suggestions, our results demonstrated that vascular alterations do not disappear within 4 wk after LDLT, regardless of the outcome. Thus, we have suggested that optimal persistence of a systemic hyperdynamic state after LDLT is necessary for successful outcomes in recipients with cirrhosis (unpublished data).

A cirrhotic systemic hyperdynamic state is symbolized by expanded BV, high CO and low TPR[3,9,32], and the preload focuses on the balance between CO and BV[46,47]. Thus, we suggest that the balance of CO to BV is an accurate estimator of the optimal stability of the characteristic systemic hyperdynamic state (unpublished data). On the other hand, to determine the systemic hemodynamic parameters related to liver transplantation, the MTT is a rigorous indicator of kinetic behavior circuits[1,9]. MTT values precisely reflect systemic hemodynamics, which are especially influenced by preload factors. That is, a greater CO is proportional to a shorter MTT, and a large BV is proportional to a prolonged MTT. Accordingly, CO/BV and MTT represent mirror images. The results presented here showed significant temporal differences between the two groups in these precise systemic hemodynamic parameters. Thus, we suggest that the recipients in Group Ⅱ showed subtle disorders of the systemic hyperdynamic state after LDLT, in contrast to the recipients in Group Ⅰ.

Other studies have focused on systemic hemodynamics or splanchnic circulation after LDLT, and some investigators have demonstrated that systemic hemodynamics are well correlated with the splanchnic circulation[41,44,48]. Interestingly, the results for the splanchnic circulatory parameters in the current study reveal that subtle disorders of the optimal systemic hyperdynamic state easily influence portal venous flow, rather than hepatic arterial flow. Vascular alterations because of portal hypertension develop in vessels that originally flow into the portal vein under normal portal pressure, and represent one of the causes of a large BV. Hence, we suggest that the imbalance between the greater CO and larger BV after LDLT in Group Ⅱ caused stagnation of the tributary blood flow in the dilated vein and collateral pathways, which resulted in a decrease in portal venous flow. It was also of interest that recipients with cirrhosis with good

outcomes (i.e., Group I) showed a clear tendency toward postoperative portal venous overflow compared with that in healthy individuals. We have previously demonstrated that the persistence of a systemic hyperdynamic state is indispensable for recipients with cirrhosis after LDLT (unpublished data), and therefore consider that excessive portal flow after LDLT seems to be correlated with a postoperative systemic hyperdynamic state. Since portal venous flow has been shown to have a large influence on liver regeneration after LDLT[49], we conclude that successful clinical outcomes in cirrhotic LDLT recipients can be attributed to optimal stability of the systemic hyperdynamic state, which yields sufficient portal venous flow. Based on our results for Group I as compared with Group Ⅱ, we suggest that continuous sufficient portal venous flow, with even a slight surplus, supported by the optimal systemic hyperdynamic state, is necessary for good outcomes after LDLT in recipients with cirrhosis. Since reversible graft damage might begin slowly from the early postoperative period, we suggest that appropriate intensive clinical management of hemodynamics will greatly impact on further improvements in LDLT outcomes.

COMMENTS

Background

Prior to undergoing LDLT, recipients with cirrhosis generally develop peculiar systemic and splanchnic hemodynamics due to portal hypertension. To ascertain correlations between systemic hemodynamics and splanchnic circulation, we performed simultaneous assessment of systemic hemodynamics and directly measured splanchnic circulation by systemic dye distribution and ultrasound.

Research frontiers

We clarify how the systemic hemodynamic state impacts on the local graft circulation in recipients with cirrhosis who underwent LDLT. Vascular alterations due to portal hypertension develop in vessels that originally flow into the portal vein under normal portal pressure, and represent one of the causes of a large BV. Hence, we suggest that the imbalance between the greater CO and larger BV after LDLT caused stagnation of the tributary blood flow in the dilated veins and collateral pathways, which resulted in a decrease in portal venous flow.

Innovations and breakthroughs

We also identified the hemodynamic state required for an excellent clinical outcome after LDLT. Since portal venous flow has been shown to have a large influence on liver regeneration after LDLT, we suggest that successful clinical outcomes in LDLT recipients with cirrhosis can be attributed to optimal stability of the systemic hyperdynamic state, which yields sufficient portal venous flow.

Applications

The methods in this study (PDD and ultrasound) are advantageous for clinical applications because of their simplicity of bedside use, rapid real-time presentation of results, and cost-effectiveness. Hence, we suggest that appropriate intensive clinical management of hemodynamics based on real-time and reliable results measured by non-invasive methods will have a large impact on further improvements in LDLT outcomes.

Terminology

Splanchnic blood flow in this study refers to that in cirrhotic recipients after living-donor liver transplantation.

Peer review

This study builds on previous observations by the same group that hyperdynamic systemic circulation persists following transplantation in patients who previously had cirrhosis, and that this is important for sustaining portal venous flow. The current manuscript focuses on the changes with respect to splanchnic hemodynamics. The authors have demonstrated significant differences in portal venous flow dynamics between a group of 25 individuals that had a good clinical outcome post-transplantation compared with five that had a poor postoperative course. This article has sufficient originality regarding the understanding of post-liver transplant hemodynamics.

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CLINICAL RESEARCH

Expression of matrix metalloproteinase-1 and tumor necrosis factor-α in ulcerative colitis

Ying-De Wang, Jing-Wei Mao

Ying-De Wang, Jing-Wei Mao, Department of Gastroenterology, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China
Correspondence to: Dr. Ying-De Wang, Department of Gastroenterology, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, China. albertwyd@yahoo.com.cn
Telephone: +86-411-83635963-3162 Fax: +86-411-83632844
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Abstract

AIM: To examine the expression of matrix metalloproteinase-1 (MMP-1) and tumor necrosis factor-α (TNF-α) in the colon mucosa of patients with ulcerative colitis (UC).

METHODS: Reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry were used to examine the expression of MMP-1 and TNF-α at both mRNA and protein levels in the colon mucosa of patients with UC. Correlation between MMP-1 and TNF-α and their correlation with the severity of the disease were also analyzed statistically.

RESULTS: The expression of MMP-1 and TNF-α in the ulcerated and inflamed colon mucosa of patients with UC was significantly higher than that in the non-inflamed mucosa of normal controls at both mRNA and protein levels. Furthermore, the expression of MMP-1 and TNF-α in the ulcerated area was significantly higher than that in the inflamed area of patients with UC (0.9797 ± 0.1433 *vs* 0.6746 ± 0.0373, 0.8669 ± 0.0746 *vs* 0.5227 ± 0.0435, $P < 0.05$). There was no statistically significant difference in the non-inflamed area of normal controls. There was a significant correlation between MMP-1 and TNF-α expression (0.9797 ± 0.1433 *vs* 0.8669 ± 0.0746, $P < 0.05$), the correlating factor was 0.877. MMP-1 and TNF-α showed a significant correlation with the severity of the disease (0.0915 ± 0.0044 *vs* 0.0749 ± 0.0032 , 0.0932 ± 0.0019 *vs* 0.0724 ± 0.0043, $P < 0.05$), their correlating factors were 0.942 and 0.890, respectively.

CONCLUSION: Excessively expressed MMP-1 directly damages the colon mucosa by degrading extracellular matrix (ECM) in patients with UC. While damaging colon mucosa, excessively expressed TNF-α stimulates MMPs secreting cells to produce more MMP-1 and aggravates the mucosa damage. MMP-1 promotes secretion of TNF-α in a positive feedback manner to cause further injury in the colon mucosa. MMP-1 and TNF-α correlate well with the severity of the disease, and therefore, can be used clinically as biological markers to judge the severity of UC.

Key words: Ulcerative colitis; Matrix metalloproteinase-1; Tumor necrosis factor-α; Reverse transcription-polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, non-specific inflammatory disease of the colon mucosa with an increasing morbidity due to life pattern changes in China. However, its etiology and pathogenesis are still unknown. Pathophysiologically, ulceration in the mucosal and submucosal areas of patients with UC is due to excessive degradation of extracellular matrix (ECM). In recent years, matrix metalloproteinases (MMPs) and some cytokines have been implicated in the development of a number of diseases, such as multiple sclerosis, rheumatic disease and UC[1-3]. In patients with UC, MMPs participate in tissue repair, vascularization and leucocyte chemotaxis in the ulcerated and inflamed colonic mucosa[3]. MMP-1 produced by cytokine-activated interstitial cells is one of the most important enzymes in degrading ECM[4]. Excessive expression of MMP-1 in the diseased colon mucosa of UC patients causes excessive hydrolysis of the ECM and ulceration[5,6]. It is also believed that imbalance between inflammatory and anti-inflammatory cytokines plays a central role in the development of UC[7]. For example, TNF-α, an important inflammatory cytokine produced by macrophages in the colon, takes part in the pathogenesis of UC[8] and can directly damage the colonic mucosal barrier, causing inflammatory changes in UC. Therefore, in this study we measured MMP-1 and TNF-α transcript and their proteins using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry to explore

their possible role and interrelationship in the pathogenesis of UC.

MATERIALS AND METHODS

Patients and samples

Thirty-six patients with UC confirmed by clinical manifestations, colonoscopy and biopsy were enrolled in this study. Among these patients, 15 were males and 21 were females with their age ranged from 22 to 72 years and averaged 44 years. Samples were taken from the ulcerated, inflamed and non-inflamed areas of the colon mucosa during colonoscopy. There were 4 patients with pan-colon lesions, 3 with hemi-colon lesions, 19 with recto-sigmoid lesions, and 10 with rectal lesions. Based on the clinical manifestations and colonoscopic findings, 8 patients were classified into mild type, 21 into moderate type, and 7 into severe type. Meanwhile, 20 normal subjects were chosen as normal controls, 12 of them were males and 8 were females with their age ranged from 22 to 56 years and averaged 34 years. Biopsy samples were immediately snap frozen in liquid nitrogen and stored at -80℃ for RT-PCR. Biopsy samples were fixed in formalin, embedded in paraffin and cut into 4 μm-thick sections for immunohistochemistry.

Total RNA extraction

Total RNA was extracted from the frozen samples using a RNA isolation kit (Invitrogen Company) following the manufacturer's instructions. Five μL of the extracted RNA was run on 1% agarose gel electrophoresis to identify the extracted products.

RT-PCR for MMP-1 and TNF-α

RT-PCR was performed using the TaKaRa RNA PCR kit 3.0 (AMV) (supplied by Dalian Baosheng Biotechnology Company) following the manufacturer's instructions. Primer sequences used are as follows: MMP-1 (sense: 5'-ATGCGAACAAATCCCTTCTACC-3', antisense: 5'-TTCCTCAGAAAGAGCAGCATCG-3'), TNF-α: (sense: 5'-CTGTAGCCCATGTTGTAGC-3', antisense: 5'-CAATGATCCCAAAGTAGACCT-3'). Primers for β-actin were used as the internal control (sense: 5'-CCTTCCTGGGCATGGAGTCCTG-3', antisense: 5'-GGAGCAATGATCTTGATCTTC-3'). Reverse transcription was carried out at 30℃ for 10 min, at 42℃ for 30 min, at 99℃ for 5 min, and at 5℃ for 5 min. PCR was performed as follows: initial denaturation at 94℃ for 2 min, followed by 35 amplification cycles at 94℃ for 30 s, at 53℃ for 30 s, at 72℃ for 1 min, extension at 72℃ for 10 min. Five μL of PCR products was run on 2% agarose gel electrophoresis.

Immunohistochemistry

Sample sections were washed 3 times with PBS, 3 min each time after initial treatment. Primary antibodies, mouse anti-human MMP-1 monoclonal antibody and rabbit anti-human TNF-α polyclonal antibody (Beijing Zhongshan Biology Company) were added and incubated at room temperature for 1.5 h, washed again and incubated with peroxidase-conjugated secondary antibody for 15 min and washed again. A brown product was developed in diaminobenzidine (DAB) for 10 min.

Result determination and statistical analysis

A bio-imaging system (PALL Company, USA) was employed to analyze the density of the bands of PCR products. MMP-1 mRNA and TNF-α mRNA were semi-quantitatively expressed by the ratios between MMP-1, TNF-α and β-actin OD values. All values were expressed as mean ± SD.

Results of immunohistochemistry were considered positive when brown particles appeared in the cells after DAB staining. An image-pro-plus 4.5 microscopic image analyzing system was used to measure the density of the positive products. Five fields in each section were randomly selected to measure the total density and area. The mean density was determined by calculating the ratio between the total density and area in each section. A bigger ratio value indicates a greater expression of the corresponding proteins.

Student-Neuman-Keuls test was used to compare MMP-1 and TNF-α: mRNAs and their corresponding proteins in different colon samples and in different severity of the disease. Spearman correlation analysis was used to study the relationship between MMP-1, TNF-α and severity of the disease. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 11.5 for windows.

RESULTS

Expression of MMP-1 and TNF-α mRNA in different colon areas of UC patients

The expression of MMP-1 and TNF-α mRNA in the ulcerated area of colon was significantly higher than that in the inflamed colon area of patients with UC and non-inflamed colon area of normal controls ($P < 0.05$). The expression of MMP-1 and TNF-α mRNA in the inflamed colon area of patients with UC was also significantly higher than that in the non-inflamed colon area of normal controls ($P < 0.05$), but the extent was not as high as that in the ulcerated area. There was no statistically significant difference in non-inflamed colon area of normal controls (Table 1, Figures 1 and 2).

Expression of MMP-1 and TNF-α mRNA in patients with different severity of UC

The expression of MMP-1 mRNA was significantly higher in different groups of patients than in normal controls ($P < 0.05$). Comparison among the three groups showed that the highest expression of MMP-1 and TNF-α mRNA was seen in the group of patients with severe UC followed by in groups of patients with mild and moderate UC (Table 2).

Correlation MMP-1 and TNF-α mRNA expression

Correlation studies showed that the expression of MMP-1 mRNA was significantly correlated with that of TNF-α mRNA. The correlating factor was 0.877 ($P < 0.01$).

Table 1 Expression of MMP-1 and TNF-α mRNA in samples from different areas of colon of UC patients (mean ± SD)

Samples	MMP-1 mRNA	TNF-α mRNA	*P* value
Ulcerated area	0.9797 ± 0.1433	0.8669 ± 0.0746	< 0.05[a,c,e]
Inflamed area	0.6746 ± 0.0373	0.5227 ± 0.0435	< 0.05[a,c]
Non-inflamed area	0.0071 ± 0.0025	0.0302 ± 0.0299	> 0.05
Normal controls	0.0062 ± 0.0029	0.0280 ± 0.0060	

[a]*P* < 0.05 *vs* non-inlamed area; [c]*P* < 0.05 *vs* normal controls; [e]*P* < 0.05 *vs* inflamed area.

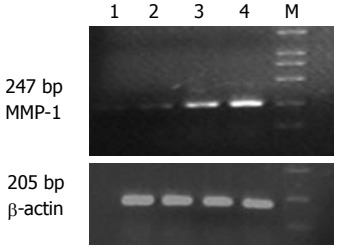

Figure 1 MMP-1 mRNA RT-PCR. Lane 1: Normal controls; lane 2: Non-inflamed area; lane 3: Inflamed area; lane 4: Ulcerated area; lane M: Marker.

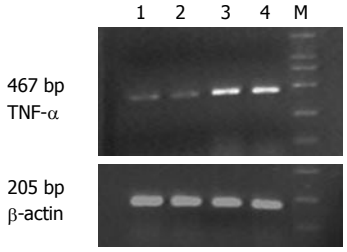

Figure 2 TNF-α mRNA RT-PCR. Lane 1: Normal controls; lane 2: Non-inflamed area; lane 3: Inflamed area; lane 4: Ulcerated area; lane M: Marker..

The expression of MMP-1 and TNF-α mRNA was also significantly correlated with the severity of the disease. The correlating factor was 0.942 and 0.890, respectively ($P < 0.01$).

Results of immunohistochemistry

Immunohistochemistry showed that the expression of MMP-1 and TNF-α in different areas of colon was identical. The expression of MMP-1 and TNF-α in the ulcerated area was significantly higher than that in the inflamed colon area of UC patients and non-inflamed colon area of normal controls ($P < 0.05$). The expression of MMP-1 and TNF-α in the inflamed colon area of UC patients was also significantly higher than that in the non-inflamed colon area of normal controls ($P < 0.05$), but it was not as high as that in the ulcerated area. There was no statistically significant difference in the non-inflamed colon area of normal controls (Figure 3A-D and Figure 4A-D, Table 3).

Protein analysis showed that the expression of MMP-1 and TNF-α in patients with different severity of the disease was identical The expression of MMP-1 and TNF-α was significantly higher in different groups than that in normal controls ($P < 0.05$). Comparison among the three groups showed that the highest expression of MMP-1 and TNF-α was seen in the group of patients with severe UC followed by in groups with mild and moderate UC (Table 4).

Table 2 Expression of MMP-1 mRNA in samples from patients with different severity of UC (mean ± SD)

Samples	MMP-1 mRNA	TNF-α mRNA	*P* value
Ulcerated area	0.9797 ± 0.1433	0.8669 ± 0.0746	< 0.05[ace]
Inflamed area	0.6746 ± 0.0373	0.5227 ± 0.0435	< 0.05[ac]
Non-inflamed area	0.0071 ± 0.0025	0.0302 ± 0.0299	< 0.05
Normal controls	0.0062 ± 0.0029	0.0280 ± 0.0060	

[a]*P* < 0.05 *vs* moderate type; [c]*P* < 0.05 *vs* severe type; [e]*P* < 0.05 *vs* normal controls.

Table 3 Expression of MMP-1 and TNF-α proteins in samples from different areas of colon of UC patients (mean ± SD)

Samples	MMP-1	TNF-α	*P* value
Ulcerated area	0.0891 ± 0.0062	0.0903 ± 0.0054	< 0.05[a,c,e]
Inflamed area	0.0791 ± 0.0047	0.0832 ± 0.0028	< 0.05[a,c]
Non-inflamed area	0.0047 ± 0.0040	0.0036 ± 0.0013	> 0.05
Normal controls	0.0048 ± 0.0016	0.0029 ± 0.0021	

[a]*P* < 0.05 *vs* non-inlamed area; [c]*P* < 0.05 *vs* normal controls; [e]*P* < 0.05 *vs* inflamed area.

Table 4 Expression of MMP-1 and TNF-α protein in samples from UC patients with different severity of the disease (mean ± SD)

Samples	MMP-1	TNF-α	*P* value
Mild type	0.0749 ± 0.0032	0.0724 ± 0.0043	< 0.05[a,c,e]
Moderate type	0.0812 ± 0.0030	0.0840 ± 0.0036	< 0.05[a,e]
Severe type	0.0915 ± 0.0044	0.0932 ± 0.0019	< 0.05[e]
Normal controls	0.0048 ± 0.0016	0.0029 ± 0.0021	

[a]*P* < 0.05 *vs* moderate type; [c]*P* < 0.05 *vs* severe type; [e]*P* < 0.05 *vs* normal controls.

DISCUSSION

Ulcerative colitis (UC) is a chronic, non-specific inflammatory disease with ulceration in the mucosal and submucosal areas of colon. Excessive degradation and insufficient synthesis of extracellular matrix (ECM) are the main pathophysiological events occurring in the process of ulceration. Since matrix metalloproteinases (MMPs) are the major hydrolytic enzymes that degrade ECM, the increased activity of MMPs is responsible for tissue damage of the colon in UC patients. It has been well accepted that inflammatory cytokines including TNF-α participate in the pathogenesis of UC[7]. The relationship between MMPs and inflammatory cytokines remains to be studied when both of them take part in the pathogenesis of UC.

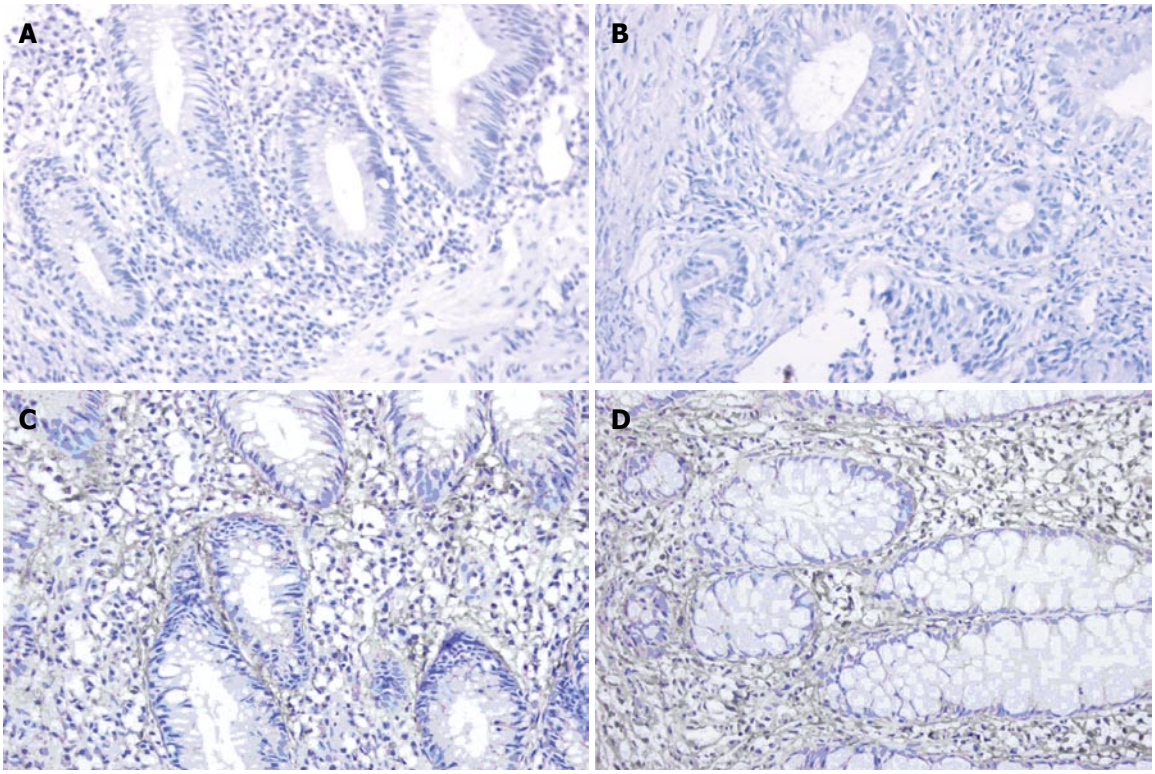

Figure 3 Expression of MMP-1 in normal controls (**A**), in non-inflamed area (**B**), in inflamed area (**C**), and in ulcerated area (**D**).

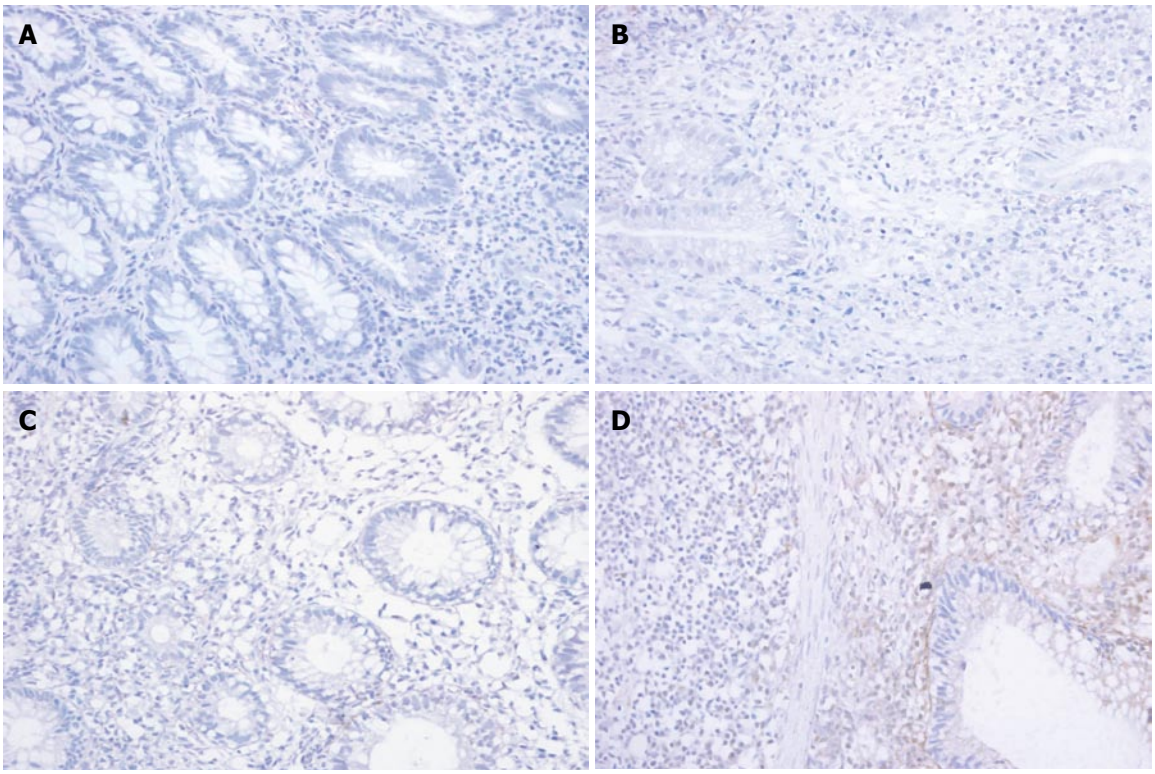

Figure 4 Expression of TNF-α in normal controls (**A**), in non-inflamed area (**B**), in inflamed area (**C**), and in ulcerated area (**D**).

MMPs are a group of zinc-dependent peptidases that degrade ECM. MMP-1, also known as interstitial collagenase, degrades mainly collagen types Ⅰ, Ⅱ, Ⅲ, Ⅵ, Ⅸ and proteoglycan, and plays an important role in degrading ECM in UC patients. Using RT-PCR and immunohistochemistry, we found that at both transcription and protein levels, the expression of MMP-1 in ulcerated and inflamed colon area of patients with UC

was significantly higher than that in non-inflamed colon area of normal controls. Furthermore, the expression of MMP-1 in ulcerated area was significantly higher than that in the inflamed area. In the present study, MMP-1 expression was closely correlated with the severity of the disease (correlating factor was 0.942, $P < 0.05$), indicating that MMP-1 is closely related to colon mucosa damage in UC patients[9,10]. Immunohistochemistry showed that MMP-1 was expressed mainly in the cytoplasm of mono-macrophages, which is consistent with the results reported by Von Lampec *et al*[11]. McKaig *et al*[12] also found that MMP-1 is expressed in damaged tissue vascular smooth muscle cells, indicating that MMP-1 may be related with formation of new blood vessels.

Our results showed that at transcription and protein levels, the expression of TNF-α in the ulcerated and inflamed area of UC patients was significantly higher than that in the non-inflamed area of normal controls. The expression of TNF-α was closely correlated with the severity of the disease (correlating factor was 0.890, $P < 0.05$), indicating that the more severe the disease, the higher the TNF-α expression. Immunohistochemistry revealed that the TNF-α positively stained cells were mainly mono-macrophages. Ishiguro[13] also reported that TNF-α expression in the diseased mucosa of colon in UC patients is significantly higher than that in the unaffected area of normal controls, suggesting that lipopolysaccharide produced by the intestinal flora may directly activate macrophages in the lamina propria, proliferating and producing a series of cytokines including TNF-α which damage the mucosa barrier of colon and produce typical inflammatory changes in UC. Apart from inflammatory cytokines, anti-inflammatory cytokines such as IL-10 also take part in the pathogenesis of UC. Gasche *et al*[14] reported that the expression of IL-10 mRNA is significantly decreased while Niessner *et al*[15] found that IL-10 mRNA is highly expressed in active UC, indicating that the expression of IL-10 mRNA is different in UC patients. Using in situ hybridization and immunohistochemical methods, Autschbach *et al*[16] showed that the number of IL-10 secreting monocytes and the mucosal expression of IL-10 are both significantly increased, but the expression of IL-10 in lanmina propria is relatively low, suggesting that IL-10 cannot effectively inhibit inflammatory cytokines such as TNF-α in lamina propria.

In the present study, MMP-1 was found to be closely correlated with TNF-α, indicating that there is a certain relationship between MMPs and cytokines. There is evidence that multiple cytokines may influence the expression of MMPs during inflammatory responses. Previous studies indicate that IL-1β and TNF-α are potent stimulators of MMP-1 and MMP-3[17,18]. They can regulate the secretion of MMP-1 and MMP-3 produced by mono-macrophages. Sylvia *et al*[19] found that the activity of T cells is correlated with the extent of colon mucosa damage, and that the colon mucosa injury is mediated by endogenously produced MMPs. Some authors believe that anti-inflammatory cytokines, such as IL-4 and IL-10, are able to inhibit the secretion of MMPs by monocytes[20-22]. Qiu *et al*[23] found that MMP-2 and MMP-9 combine with CD44 receptors on the cell membrane to form MMP-1/19-CD44 complex, making the inactivated TGF-β become its active form through hydrolysis and carry out its biological functions. Black *et al*[24] reported that MMPs activate TNF-α on cell membrane through hydrolysis to make it in an active state. MMPs may also block some cytokines, such as IL-6 and TGF-α to down-regulate their activities[25]. It is believed that MMPs not only appear in the down stream of inflammatory responses but also exert a positive feedback effect on cytokines. Therefore, they can be regarded as important "regulators" of inflammatory responses.

MMPs and cytokines play an important role in the process of UC. When infection, diet or other environmental factors act on hereditarily susceptible individuals, abnormal immune responses of the intestine may activate immune cells (such as T cells, lymphocytes and macrophages) to secrete a big amount of cytokines, inflammatory mediators and complements. These substances directly damage the colon mucosa, and induce interstitial cells (including smooth muscle cells, fibroblasts and mono-macrophages) to secrete MMPs. The increased MMPs degrade ECM in the colon mucosa, leading to mucosa damage and ulceration. While cytokines influence MMPs expression, and MMPs themselves are able to up-regulate cytokines through certain ways to cause further damage on the colon mucosa, MMPs can be inhibited by their inhibitors (MMPI) including their natural ones[26], revealing that MMPs have become one of the targets in anti-inflammatory treatment. MMPs inhibitors used in treatment of malignant tumors in clinical phase Ⅲ trial[27] in America and Europe can also be used in the treatment of patients with UC[7], while anti-inflammatory or inflammatory cytokine inhibitors can be used to reduce MMPs expression so as to indirectly reduce tissue damage and ulceration in UC patients. For example, a TNF-α antagonist, Infliximab, has been proved effective against adult and children UC patients[28,29].

In conclusion, excessively expressed MMP-1 directly damages the colon mucosa by degrading ECM in UC patients. While damaging colon mucosa, excessively expressed TNF-α stimulates MMPs secreting cells to produce more MMP-1 and aggravates the mucosa damage. MMP-1 promotes secretion of TNF-α in a positive feedback manner to cause further injury in the mucosa of colon. MMP-1 and TNF-α can be used clinically as biological markers to judge the severity of UC.

COMMENTS

Background

Matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs) and inflammatory cytokines, e.g., tumor necrosis factor-α (TNF-α) participate in the development of ulcerative colitis (UC) which is a chronic, non-specific inflammatory disease of the colon mucosa with unknown etiology and pathogenesis. This study was to deal with the expression of MMP-1 and TNF-α transcript and their proteins in colonic mucosa of patients with UC and their interrelationships in the pathogenesis of UC.

Research frontiers

Participation and functions of MMPs, TIMPs and inflammatory cytokines in the

pathogenesis of UC have been extensively studied in recent years. Study in this field has become one of the hotspots at present. Previous studies have demonstrated that MMPs and some inflammatory cytokines, such as TNF-α, are responsible for the development of ulceration and inflammation in the colonic mucosa of UC patients. Based on these findings, treatment targeting these proteins, such as anti-TNF-α antibody and exogenous MMPs inhibitors has been designed and studied in animal models. Preliminary results of these studies have shown beneficial and promising effects. Further experimental and clinical studies are needed before certain conclusions can be reached.

Innovations and breakthroughs

The association between MMPs and inflammatory cytokines with UC has been studied previously. However, most of the studies focused on their functions on the development of UC. The relationship between MMPs and other cytokines and the activity of UC remains largely unexplored. This study has bridged this gap and may provide additional targets for therapeutic development.

Applications

Since some basic evidence provided for MMP-1, TNF-α and their relationships in the development of UC, therapeutic approaches targeting MMPs or TNF-α, can be implemented in future study and new methods for treating UC may be developed.

Terminology

Matrix metalloproteinases (MMPs): MMPs are a group of zinc-dependent peptidases that degrade extracellular matrix (ECM). In this family, more than 20 MMPs have been identified. MMP-1, also known as interstitial collagenase, degrades mainly collagen type Ⅰ, Ⅱ, Ⅲ, Ⅵ, Ⅸ, and proteoglycan, and plays an important role in degrading ECM and in leading to colonic mucosa damages in UC patients.

Peer review

This is an informative study demonstrating the association between metalloproteinase (MMP) and tumor necrosis factor (TNF) with disease activity in individuals with ulcerative colitis. The association of TNF with UC is well known but the relationship of other cytokines with disease activity remains largely unexplored. This study is an attempt to bridge this gap and may provide additional targets for therapeutic development. The preliminary conclusion is justified and substantiated by the results obtained.

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RAPID COMMUNICATION

Small caliber overtube-assisted colonoscopy

Shai Friedland, Roy M Soetikno

Shai Friedland, Roy M Soetikno, Stanford University School of Medicine and VA Palo Alto Health Care System, United States
Correspondence to: Shai Friedland, MD, Department of Gastroenterology, VA Palo Alto. 3801 Miranda Avenue, Palo Alto, CA 94304, United States. shai_friedland@yahoo.com
Telephone: +1-650-7877099 Fax: +1-650-3217917
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Abstract

AIM: To combine the benefits of a new thin flexible scope with elimination of excessive looping through the use of an overtube.

METHODS: Three separate retrospective series. Series 1: 25 consecutive male patients undergoing unsedated colonoscopy using the new device at a Veteran's hospital in the United States. Series 2: 75 male patients undergoing routine colonoscopy using an adult colonoscope, pediatric colonoscope, or the new device. Series 3: 35 patients who had incomplete colonoscopies using standard instruments.

RESULTS: Complete colonoscopy was achieved in all 25 patients in the unsedated series with a median cecal intubation time of 6 min and a median maximal pain score of 3 on a 0-10 scale. In the 75 routine cases, there was significantly less pain with the thin scope compared to standard adult and pediatric colonoscopes. Of the 35 patients in the previously incomplete colonoscopy series, 33 were completed with the new system.

CONCLUSION: Small caliber overtube-assisted colonoscopy is less painful than colonoscopy with standard adult and pediatric colonoscopes. Male patients could undergo unsedated colonoscopy with the new system with relatively little pain. The new device is also useful for most patients in whom colonoscopy cannot be completed with standard instruments.

Key words: Colonoscopy; Endoscopy; Colon Cancer; Colon cancer screening

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INTRODUCTION

Colonoscopy is typically performed using relatively large-diameter (11-13 mm) pediatric and adult instruments with enough rigidity to permit advancement of the instrument despite multiple turns within the bowel[1-3]. With these instruments, looping of the endoscope is a common difficulty that results in pain for the patient and hinders advancement of the endoscope[4,5]. In an effort to overcome looping, which is particularly common in the sigmoid colon, some practitioners have used stiffening overtubes that are preloaded on the back end of the scope and advanced over the colonoscope after negotiation of the sigmoid colon[6-8]. With the tube in place, further advancement of the instrument can be attained with minimal looping in the sigmoid; the overtube facilitates transmission of force from the endoscopist's pushing hand to the proximal end of the overtube. However, the overtubes employed for colonoscopy in the past have been relatively bulky and rigid devices that accommodate the large diameter of standard colonoscopes.

It is sometimes possible to perform colonoscopy using relatively thin and flexible upper endoscopes[9]. Thinner, more flexible scopes are often more easily advanced through the left colon[10]; this is perhaps the major reason why many endoscopists prefer pediatric colonoscopes over standard adult colonoscopes in female patients and in patients with sigmoid adhesions[2]. However, even pediatric colonoscopes are often associated with more difficulty in advancement through the proximal colon due to excessive looping[2]. These observations suggest that a very thin and flexible scope might facilitate insertion through the distal colon, but a mechanism to prevent excessive looping is important for optimal advancement through the proximal colon. One alternative to conventional colonoscopy that employs this strategy is to perform the procedure using a double balloon enteroscope[11-13]. The double balloon system also employs a very thin scope and an overtube, with the addition of balloons on the scope tip and overtube tip that can be inflated to secure the position by pressing against the bowel wall[14-16]. The double balloon system is used increasingly in patients who have failed conventional colonoscopy, but a major limitation is that the procedure is laborious and time consuming[17-19]. We surmised that by using a standard 160 cm length of scope, rather than the 200 cm long double balloon enteroscope, and a short 60 cm overtube, rather than a 140 cm long double balloon overtube, the procedure would be more efficient.

MATERIALS AND METHODS

The new colonoscopy system consists of a thin 9 mm scope, 170 cm in length, together with a 13 mm diameter 60 cm long overtube. The new 9 mm endoscope has the same outer diameter and instrument channel diameter (2.8 mm) as diagnostic upper endoscopes, but a 170 cm length that is similar to that of standard colonoscopes. The new scope has already received regulatory approval by the U.S. Food and Drug Administration for routine clinical use. The endoscope was provided by the Olympus corporation (Olympus America, Melville, New York, USA). The overtube (TS-13140, Fujinon Corporation, Wayne, New Jersey, USA) has a proprietary coating that reduces friction with the scope when the system is exposed to water; it is available commercially and is widely used in double balloon endoscopy. Because the overtube was too long, we cut off the proximal (near the hub) 100 cm and moved the plastic handle from its original position to the proximal end of the shortened tube (Figure 1). We also removed the inflatable latex balloon at the tip of the overtube because our earlier experience suggested that it is not generally helpful. Prior to each procedure, the overtube was temporarily filled with water to activate the lubrication system inherent in the tube and then back-loaded to the hub of the endoscope, leaving the distal 110 cm of the endoscope free for performing the initial portion of the examination without the overtube in place. After reaching the transverse colon, the scope was reduced, and the overtube was advanced over the scope until the handle on its proximal end was near the buttocks. An assistant then held the handle on the end of the overtube and the scope was advanced to the cecum.

This study consists of 3 retrospective series of patients undergoing colonoscopy at the Veterans Affairs Palo Alto Health Care System. Informed consent was obtained from all patients. The study was approved by the institutional review board of our hospital. All of the procedures were done by a single endoscopist with 8 years of experience performing approximately 1000 colonoscopies per year. The first series consisted of 25 consecutive male patients who were scheduled for unsedated colonoscopy (no medications given for the procedure); the patients were scheduled for unsedated procedures because of patient preference, medical contraindications to sedation, or lack of a driver to take them home after the procedure. The second series consisted of 75 consecutive male patients undergoing routine colonoscopy (3 female patients, 3 patients with previous partial colectomy and 1 patient with inflammatory bowel disease who necessitated a high-resolution magnification scope were not included in the series). An adult (Olympus CF-Q160AL), pediatric (Olympus PCF-Q180AL) and the thin scope/overtube were used in alternating cases. Patients were pre-medicated with lorazepam 2 mg sublingually (1 mg for patients over age 80) 15 min before the procedure. Patients were instructed by the nursing staff to request additional medication if they experienced pain or discomfort. Intravenous fentanyl was administered if the patient requested further sedation. The third series consisted of 35 patients who had incomplete colonoscopies in our endoscopy unit (the cecum was not reached) using any combination of standard adult (Olympus CF-Q160AL) and/or pediatric (Olympus PCF-160AL or PCF-Q180AL) endoscopes. The incomplete colonoscopies were performed by one of eight experienced attending endoscopists who work in our department.

Figure 1 The new 9 mm scope is shown alongside the 60 cm-long overtube.

Statistical analysis

Statistical comparison calculations were performed with two-tailed unequal-variance student's *t*-test[20]. Odds ratios and confidence intervals were calculated with the Newscombe-Wilson method without continuity correction[21].

RESULTS

In the first series, unsedated colonoscopy was successful in 25 consecutive patients at the Veterans Affairs Palo Alto Health Care System using the new device. None of the patients received any medication for the procedure. The indication for colonoscopy was a previous history of adenoma in 14 patients, positive stool occult blood in 3, screening in 2, family history of colon cancer in 2, hematochezia in 2, anemia in 1 and constipation in 1. Patients underwent unsedated colonoscopy for one of three reasons: patient preference (10 patients), inordinately high sedation risk (6) or unavailability of a driver to take them home after receiving sedation (9). All of the patients were male veterans. The age of the patients ranged between 53 and 94, with an average age of 68.1 and a median of 70.

Cecal intubation was achieved in all 25 patients, in a median time of 6 (average 6.4, range 2.5-15) min. Patients rated their maximal pain level during the procedure on a 0-10 scale. The median maximal pain level was 3 (average 2.9, range 0-6.5). Six patients had a maximal pain of 4 or higher. The entire procedure lasted a median time of 13 (average 13.6, range 7-28) min, including at least one snare polypectomy in 8 patients and forceps biopsy in another 2 patients. Small (< 10 mm) areas of mild erythema from passage of the overtube were seen occasionally on withdrawal, but no mucosal disruptions or other signs of trauma were observed. There was one complication:

bleeding one week after endoscopic mucosal resection of a 1.5 cm flat adenoma. The patient underwent urgent colonoscopy with successful clipping of an actively bleeding vessel at the resection site. He did not require blood transfusion or hospitalization.

The second series consisted of 75 male patients undergoing routine screening or surveillance colonoscopy. A standard adult colonoscope, pediatric colonoscope, and the thin scope/overtube system were used alternatingly; 25 procedures were performed with each type of scope. The median age of the thin scope group was 70 ± 10, compared to 69 ± 9 in the adult scope group (P = NS). The median age of the pediatric scope group was 65 ± 8, which was significantly younger than the thin scope group (P = 0.03).

Following premedication with lorazepam, 24/25 procedures with the thin scope were completed without additional sedation medication, compared to 9/25 with the adult scope (odds ratio 43, $P < 0.005$) and 14/25 with the pediatric scope (odds ratio 19, $P < 0.01$). The mean dose of fentanyl (μg) used was 12 ± 60 with the thin scope, compared to 51 ± 53 with the adult scope ($P < 0.05$) and 39 ± 53 (P = NS) with the pediatric scope. The median maximal pain during the procedure on a 0-10 scale was 3.5 ± 2 in the thin scope group, compared to 8 ± 2 in the adult colonoscope group ($P < 0.001$), and 7.5 ± 2.5 in the pediatric colonoscope group ($P < 0.001$). The cecum was reached in all patients, but the adult colonoscope was exchanged for a smaller diameter scope in 2 patients due to acute angulation in the sigmoid, and the pediatric colonoscope was exchanged for another scope in 2 patients due to excessive looping. The median time in minutes to reach the cecum was 5.5 ± 2.5 in the thin scope group, compared to 6.0 ± 2.1 in the adult colonoscope group (P = NS), and 4.0 ± 1.9 min in the pediatric colonoscope group (P = 0.004).

In the third series, 35 patients who had previously undergone unsuccessful colonoscopy (with inability to reach the cecum) had the procedure repeated using the new device. The reasons given by the endoscopist for the inability to reach the cecum were: excessive looping (22 patients), acute sigmoid angulation (11 patients) and acute angulation at the splenic flexure (2 patients). 28 of the patients were male and 7 were female. The age ranged between 33 and 90, with a median age of 65 and a standard deviation of 13. The procedure was successful in 33; the cecum could not be reached in 2 male patients due to excessive looping and double balloon colonoscopy was successfully performed in both of these cases. The median time to reach the cecum in the 33 successful cases was 7 (standard deviation 3.9) min. The median total colonoscopy time, including snare polypectomies in 8 patients and forceps biopsies in 3 patients, was 15 (standard deviation 8.4) min. There were no complications.

DISCUSSION

Sedation practices for colonoscopy vary widely across the world; unsedated colonoscopy is commonly performed in Asia and Finland[2], whereas it is generally very poorly accepted in the United States[22-25]. A major reason is pain due to looping of the endoscope. Small caliber overtube-assisted colonoscopy can potentially decrease looping and pain enough to make unsedated colonoscopy feasible in the general population. The small caliber scope used in this study was easily and rapidly advanced through the distal colon with minimal pain. After reduction of the scope, the thin low-friction overtube was advanced into position without significant resistance. With the overtube in place, it was generally possible to directly advance the endoscope to the cecum with relatively little attention to subsequent loop formation or paradoxical backward motion of the tip upon insertion. Our study suggests that this colonoscopy system could potentially make colonoscopy without intravenous sedation feasible a significant number of patients. The thin scope/overtube system was significantly less painful than conventional adult or pediatric colonoscopes. The 25 patients who required unsedated colonoscopy for a variety of indications all had successful procedures, and only 6 had a maximal pain level of 4 or higher on a 10 point scale. In the second patient series, when routine colonoscopy was performed after premedication with sublingual lorazepam, only 1 of 25 patients in the thin scope/overtube group requested additional sedation, compared to 11 of the patients with the pediatric colonoscope and 16 with the adult colonoscope. This suggests that most male patients undergoing routine screening or surveillance colonoscopy do not require intravenous conscious sedation and would be satisfied with a mild sedative that can be administered by mouth without an intravenous line. This could potentially result in a substantial cost savings by eliminating the need for extensive monitoring of patients receiving conscious sedation, and potentially make colonoscopy feasible for many patients in an office setting.

The thin scope/overtube system offers several benefits compared to standard colonoscopes. The thin scope is generally easily advanced through the sigmoid colon, as demonstrated by the successful performance of colonoscopy in 11 patients in the third series in whom previous colonoscopy was unsuccessful due to acute sigmoid angulations. Once the scope has been advanced through the left colon and reduction of loops has been performed, the overtube is advanced into position and subsequent looping of the scope during advancement through the right colon should theoretically be minimized. We did not specifically measure looping in the procedures we performed, but in our experience once the overtube was in place the scope was easily advanced through the right colon with little effort or attention required to prevent or reduce loops. The median time required to reach the cecum was 6 min in the unsedated group and 5.5 min in the lorazepam premedication group. This suggests that despite the additional step of positioning the overtube, reaching the cecum with the system can still be in an acceptable period of time. The median overall procedure time was 13 and 13.5 min in the unsedated and lorazepam groups, including at least one snare polypectomy in approximately 1/3 of the patients, demonstrating that withdrawal and polypectomy can also be performed efficiently.

Of the 35 patients who had previously failed colonoscopy using standard instruments, 33 had a

successful procedure with the thin scope/overtube system. The median time to reach the cecum in those 33 patients was only 7 ± 3.9 min. Although these cases were subjectively more difficult than routine cases, the patients received conscious sedation, which may facilitate rapid advancement, resulting in a similar overall time to cecum as in unsedated routine cases. This compares very favorably to our prior published experience of using a double balloon enteroscope to successfully complete 19 of 20 patients with previously incomplete colonoscopies, where the median time to reach the cecum was 28 ± 20 min[26]. Based largely on this difference in time, our preference is currently to use the thin scope/overtube system in all cases after failed colonoscopy with standard instruments, and reserve the double balloon enteroscope for those situations when the thin scope/overtube system is unsuccessful.

There are clear limitations to the current study: the retrospective design, the relatively small number of patients in each of the series, the overwhelmingly male patient population, the previously documented tolerance of male American veterans to unsedated colonoscopy[2,27], and the single-center design. Since the study was retrospective, the routine screening colonoscopy patients were not randomized to the new scope or a standard adult or pediatric scope, but rather the scopes were alternated. There were no complications attributable to the thin scope/overtube system in our study (the lone complication in the 3 retrospective series was a post-polypectomy bleed in one of the unsedated patients), but all of the procedures were performed by one experienced endoscopist and it remains to be demonstrated that the system is safe when used by practitioners of varying experience. Given the substantial differences across different institutions and different countries in the performance of unsedated colonoscopy, it is difficult to predict what effect this system could have on colonoscopy practice, but our study does demonstrate the potential for making colonoscopy less painful and better tolerated without dramatically increasing procedure time or complexity.

There are several disadvantages to the small caliber endoscope and overtube system used in this study. The overtube is marketed for single-use and is expensive in its current form (approximately US$200 at our institution); shortening the tube is also cumbersome. It is conceivable that a more reasonably priced short tube could be manufactured or that a reusable version could be developed. The 9-mm scope has a relatively small 2.8-mm channel which is adequate for typical maneuvers such as snare polypectomy and clip placement, but can limit suctioning of stool residue and resected polyps. A water jet port for efficient lavage is not available. The field of view, lighting and optical resolution may be slightly compromised compared to the latest generation of high-resolution adult colonoscopes. However, the potential for reducing pain may outweigh any of these disadvantages. Further studies will also need to address whether some colonoscopies are more difficult with this system, whether there is any increase in the rate of missed lesions, and whether certain therapeutic cases would be better served by using a standard colonoscope. The ultimate goal of reducing pain during colonoscopy enough to make unsedated colonoscopy better tolerated, thereby eliminating both complications due to sedation as well as an estimated 40% of the cost of the procedure[2], is particularly important given the current widespread screening practices in many countries. Additional adjunctive measures, such as using carbon dioxide instead of air for insufflation[28,29], may also play a role in achieving this goal.

COMMENTS

Background

Colonoscopy using standard instruments is often relatively painful and most procedures are done using intravenous sedation. Reduction of pain is a major focus of research because the potential for eliminating conscious sedation may make the procedure safer and less expensive.

Research frontiers

The development of new types of scopes for performance of colonoscopy with less pain and less sedation is a major area of research. Thinner scopes can potentially cause less pain during colonoscopy, but they can also result in more loop formation which can hamper the procedure.

Innovations and breakthroughs

In this article we describe our experience using a new thin scope in combination with an overtube designed to minimize loop formation. We demonstrate that the new system is less painful than standard colonoscopes.

Applications

This study suggests that the combination of a thin scope and an overtube can be useful for unsedated, routine and difficult colonoscopies.

Terminology

Looping: the process where the scope tip does not progress forward when the endoscopist pushes the scope into the patient, but rather the mid-section of the scope bows out, resulting in stretching of the colon.

Peer review

This is an important and well written contribution. Through retrospective comparative study, the authors concluded that small caliber overtube-assisted colonoscopy is less painful than colonoscopy with standard adult and pediatric colonoscopes. Male patients can undergo unsedated colonoscopy with the system with relatively little pain. The new device is also useful for most patients in whom colonoscopy cannot be completed with standard instruments.

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RAPID COMMUNICATION

Comprehensive screening for *reg1α* gene rules out association with tropical calcific pancreatitis

Swapna Mahurkar, Seema Bhaskar, D Nageshwar Reddy, G Venkat Rao, Giriraj Ratan Chandak

Swapna Mahurkar, Seema Bhaskar, Giriraj Ratan Chandak, Genome Research Group, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India
D Nageshwar Reddy, G Venkat Rao, Asian Institute of Gastroenterology, Punjagutta, Hyderabad 500 082, India
Supported by the Council of Scientific and Industrial Research, Ministry of Science and Technology, Government of India
Correspondence to: Giriraj Ratan Chandak, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India. chandakgrc@ccmb.res.in
Telephone: +91-40-27160222-41 Fax: +91-40-27160591
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Abstract

AIM: To investigate the allelic and haplotypic association of *reg1α* gene with tropical calcific pancreatitis (TCP). Since TCP is known to have a variable genetic basis, we investigated the interaction between mutations in the susceptibility genes, *SPINK1* and *CTSB* with *reg1α* polymorphisms.

METHODS: We analyzed the polymorphisms in the *reg1α* gene by sequencing the gene including its promoter region in 195 TCP patients and 150 ethnically matched controls, compared their allele and haplotype frequencies, and their association with the pathogenesis and pancreaticolithiasis in TCP and fibro-calculous pancreatic diabetes.

RESULTS: We found 8 reported and 2 novel polymorphisms including an insertion-deletion polymorphism in the promoter region of *reg1α*. None of the 5′ UTR variants altered any known transcription factor binding sites, neither did any show a statistically significant association with TCP. No association with any *reg1α* variants was observed on dichotomization of patients based on their N34S *SPINK1* or L26V *CTSB* status.

CONCLUSION: Polymorphisms in *reg1α* gene, including the regulatory variants singly or in combination with the known mutations in *SPINK1* and/or *CTSB* genes, are not associated with tropical calcific pancreatitis.

Key words: Tropical calcific pancreatitis; Lithostathine; Stone formation; Polymorphism; Haplotype

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INTRODUCTION

Chronic Pancreatitis (CP) is a continuing or relapsing inflammatory process of the pancreas resulting in exocrine and/or endocrine insufficiency. The cardinal manifestations of CP are pain, steatorrhoea, formation of pancreatic stones, and diabetes mellitus. Recently, mutations in cationic trypsinogen (*PRSS1*)[1], the serine protease inhibitor, Kazal type 1 (*SPINK1*)[2], and cystic fibrosis transmembrane regulator (*CFTR*)[3] genes have been found to be associated with chronic pancreatitis. Tropical calcific pancreatitis (TCP) is an idiopathic, juvenile, nonalcoholic form of chronic pancreatitis with a unique tropical distribution, while fibro-calculous pancreatic diabetes (FCPD) is a condition characterized by the development of diabetes secondary to TCP. A genetic etiology for TCP and FCPD was suggested by Pitchumoni *et al*[4] and confirmed by Mohan *el al*[5], who showed familial aggregation of FCPD with evidence of vertical transmission in some families. We previously reported evidence of its genetic nature, based on clustering of TCP in a few families and its association with SPINK1 mutations[6].

In a previous study we had shown that mutations in *PRSS1* did not play a role in TCP, whereas mutations in *SPINK1* gene were found in the majority of such patients[7]. Recently, we have demonstrated that mutations in pro-peptide region of cathepsin B (*CTSB*) gene are strongly associated with TCP[8]. Irrespective of mutations in different genes, premature intra-pancreatic activation of trypsinogen is believed to play a central role in the pathogenesis of chronic pancreatitis. However, the phenomenon of stone formation continues to be poorly understood. Although various hypotheses have been proposed for stone formation, the development of protein plugs appears to be an important initiating event[9]. It has been proposed that if concentration-dependent precipitation is the cause of protein plug formation, there should be an associated increase in the concentration of some proteins in the pancreatic juice[10].

Lithostathine C was initially isolated as a major

proteic component of pancreatic stones in alcoholic calcifying chronic pancreatitis, and was consequently called pancreatic stone proteic (PSP)[9]. Human PSP or Reg protein is encoded by *reg1α* gene (regenerating gene)[11] as a 166 amino acid pre-proprotein with a 22-residue long signal sequence. A similar protein with 89% homology with PSP is coded by another gene *reg1β* belonging to the same type 1 subclass but has never been isolated and its expression in pancreas remains controversial[12]. Only the Reg1α protein is highly represented in the human pancreatic secretions[13] and is found to be 100% identical to a glycoprotein that is generated by trypsin cleavage resulting in a 133 aa polypeptide previously named pancreatic thread protein (PTP). The mature protein is a soluble glycoprotein existing under 11 isoforms (17-22 kDa)[14], generated by post-translational modification such as glycosylation. Of these isoforms, S2-S5 are believed to inhibit calcite crystal growth *in vitro* and thus stone formation[15,16]. PSP is highly susceptible to trypsin cleavage at Arg11-Ile12 bond resulting in PTP formation, which is known to form fibrilla at neutral pH and is found in protein plugs or stones extracted from pancreatic ducts of CP patients[9,17]. The exact function of Reg1α protein is not clear, but it could stimulate the regeneration and/or growth of pancreatic β-cell[18]. We hypothesized that mutations in the promoter region of *reg1α* may lead to altered expression of the protein. Alternatively, variants in the coding region could predispose the Reg1 protein to increased tryptic cleavage resulting in greater formation of PTP. This may cause precipitation of PTP and obstruction of the pancreatic duct secondary to protein plugs and calculi, resulting in pancreatitis. Since high levels of intrapancreatic trypsin produced both by known mechanisms like *PRSS1* mutations or by as yet unknown mechanisms such as mutations in *SPINK1* and *CTSB* genes is an established fact, it can be speculated that intrapancreatic trypsin may cleave the soluble lithostathine (PSP S2-S5) into insoluble PTP. FCPD is a condition characterized by the development of diabetes secondary to TCP, however, the etiology of diabetes in these patients is not clear, hence we investigated the role of these polymorphisms in the pathogenesis of FCPD. Since, N34S *SPINK1* mutations occur in the majority of these patients and it is not clear whether pancreatitis is the cause or the effect of ductal obstruction, we attempted to investigate the interaction between N34S *SPINK1* mutation and L26V *CTSB* mutations and *reg1α* gene polymorphisms. We also performed haplotype analysis to see if a particular *reg1α* haplotype is associated with the disease.

MATERIALS AND METHODS

Patients and controls

195 unrelated subjects belonging to Australoid ethnicity[19] (134 males and 61 females), diagnosed with tropical calcific pancreatitis at the Asian Institute of Gastroenterology, Hyderabad and 150 age and sex matched individuals (98 males and 52 females) of the same ethnicity but without any evidence of pancreatitis on imaging studies were included as patients and controls respectively[7]. Both the patients and the controls completed a detailed questionnaire and underwent similar investigations including imaging studies. Written informed consent was obtained from all the patients and controls, before the collection of blood samples. The Institutional Ethics Committee of both participating institutes approved the study as per the guidelines of the Indian Council of Medical Research for research on human subjects.

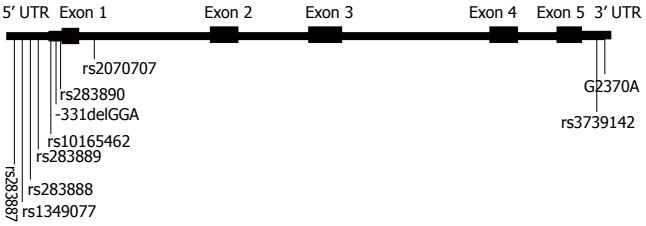

Figure 1 Diagrammatic representation of the *reg1α* gene showing exons (translated), UTRs (untranslated regions) and the location of the polymorphisms studied (constructed on the lines of *reg1α* gene structure as on UCSC genome browser, figure not to scale).

Table 1 Primer sequences and PCR conditions for the *reg1α* gene

Primer	Sequence (5'-3')	T_{ann} (℃)
1F	TGTCCCAATTCATATACTTA	50
1R	GCATGTTAGAGACGCCCTTC	
2F	CGGGAAAAGGCTCGTACTGG	60
2R	TCAGTTCTCCACCCCATTAG	
3F	TAAAAGGGAAACTGGAGACT	56
3R	CCTCCTTCTTACTTCTCAAA	
4F	TGCACTGTAGATGATTGGAG	62
4R	AAAGACTGGGGTAGGTAAAACT	
4F-INT1	TCTTGGTGGAATACAGTTAA	Seq
4F-INT2	AATGGATGTTTGGTTTTTGT	Seq

F: Forward; R: Reverse; T_{ann}: Annealing temperature; INT: Internal primer for sequencing.

Genetic analysis

Genomic DNA was isolated from patients and healthy volunteers using salting out method[20]. The human *reg1α* gene is located on 2p12 with six exons (5 translated exons, Figure 1) spanning 2962 base pairs and is known to contain TATA and CCAAT box-like sequences that are located at 27 and 100 bp upstream from the transcriptional initiation site[21]. Using the software tool Transplorer (Biobase Biological Databases, Wolfenbuttel, Germany), we attempted to identify transcription factor binding sites in a sequence of about 1600 bases upstream of transcriptional start site, which included the above-mentioned sequence[22]. We screened the complete *reg1α* gene including its exons, introns and 5'- and 3'- untranslated regions by direct sequencing, using 4 sets of primers in 50 patients and 50 controls (Table 1). PCR products were purified and sequenced individually on both the strands using Big-dye terminator cycle sequencing ready kit (Applied Biosystems, Foster City, CA) on an ABI3730 Genetic Analyzer (Applied Biosystems). In case of unclear sequence data, we repeated sequencing under various conditions until the genotype was determined correctly. Six SNPs (Table 2) that

Table 2 Distribution of polymorphisms in *reg1α* gene in patients with tropical calcific pancreatitis and healthy controls

Polymorphism[2]	rs number	Position[5]	Minor allele frequency		OR (95% CI)	*P* Value
			Patients (*n* = 195)	Controls (*n* = 150)		
G-974C[4]	rs283887	79200522	0.01	0.02	0.49 (0.02-7.10)	1.00[1]
G-938A	rs1349077	79200558	0.34	0.33	1.05 (0.56-1.96)	0.88
T-912G	rs283888	79200584	0.49	0.50	0.94 (0.54-1.78)	0.84
G-501A[4]	rs283889	79200995	0.01	0.02	0.49 (0.02-7.10)	1.00[1]
T-385C	rs10165462	79201111	0.32	0.29	1.15 (0.60-2.20)	0.65
-331delGGA[3]	-	-	0.01	0.01	1.00	1.00[1]
T-243G	rs283890	79201253	0.34	0.35	1.09 (0.75-1.58)	0.63
G209T	rs2070707	79201704	0.20	0.17	1.29 (0.78-2.12)	0.29
G2199A	rs3739142	79203694	0.34	0.34	1.01 (0.70-1.48)	0.94
G2370A[3,4]	-	-	0.01	0.03	0.33 (0.01-3.60)	0.61[1]

AA: Amino acid; OR: Odds ratio; CI: Confidence interval; [1]Yates corrected P value; [2]Nomenclature as per NCBI sequence Accession No. NT_022184; [3]Novel polymorphism; [4]Data from 50 patients & 50 controls; [5]Chromosomal location according to UCSC Genome Browser, March 2006 build (dbSNP build 126).

Table 3 Comparison of *reg1α* gene polymorphisms in FCPD and TCP patients, and controls

SNP[1]	Minor allele frequency			FCPD *vs* TCP		FCPD *vs* Controls		TCP *vs* Controls	
	FCPD (*n* = 94)	TCP (*n* = 101)	Controls (*n* = 150)	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
G-938A	0.36	0.32	0.33	1.20 (0.64-2.24)	0.55	1.14 (0.61-2.13)	0.66	0.96 (0.51-1.80)	0.88
T-912G	0.47	0.51	0.50	0.85 (0.47-1.54)	0.57	0.89 (0.49-1.60)	0.67	1.04 (0.58-1.88)	0.89
T-385C	0.31	0.30	0.29	1.05 (0.55-2.00)	0.88	1.10 (0.57-2.11)	0.76	1.05 (0.55-2.02)	0.88
T-243G	0.33	0.35	0.35	0.91 (0.49-1.71)	0.77	0.91 (0.49-1.71)	0.77	1.00	1.00
G209T	0.24	0.17	0.17	1.54 (0.73-3.27)	0.22	1.54 (0.73-3.27)	0.22	1.00	1.00
G2199A	0.41	0.33	0.34	1.41 (0.76-2.62)	0.24	1.35 (0.73-2.50)	0.31	0.96 (0.51-1.79)	0.88

SNP: Single nucleotide polymorphism; n: Number of individuals; OR: Odds ratio; CI: Confidence interval; TCP: Tropical calcific pancreatitis; FCPD: Fibrocalculous pancreatic diabetes; [1]Only SNPs with > 3% minor allele frequency have been presented; The minor allele frequency at each polymorphism was compared between the three groups and *P* value with OR and 95% CI were calculated.

exceeded allele frequency of 3% were screened in another 145 patients and 100 controls from the same ethnic background. N34S and L26V mutations in the *SPINK1* and in *CTSB* genes respectively were analyzed using the methodology as described previously[7,8]. Ten percent of randomly chosen samples were re-genotyped for validation of the data, and no genotyping error was noted.

Statistical analysis

The allele and genotype frequencies were calculated for each polymorphism (Table 2) in the whole cohort as well as in TCP and FCPD patients separately (Table 3). We analyzed any deviation from the Hardy-Weinberg equilibrium, and observed the expected genotype frequencies by Markov simulation based goodness of fit test using Arlequin software version 2[23]. Pearson's Chi-square and Yates corrected chi-square test were used to analyze the statistical significance of the difference in allelic distribution of polymorphisms in patients and controls. Haplotypes were generated with 6 polymorphisms having a minimum allele frequency greater than 3% with the accelerated Expectation-maximization algorithm using Haploview software (Version 3.2) and compared the results between patients and controls[24]. This study was 90% powered to detect a relative risk of 1.60 (http://www.dssresearch.com/). Unless indicated specifically, a *P*-value of 0.05 was considered significant in all the analyses. Chi-square, genotype relative risk, odds ratio and confidence interval were calculated using the PEPI (Programme for EPIdemiologists, ver 4.04) and DeFenetti programs (http://www.ihg.gsf.de/cgi-bin/hw/hwa1/).

RESULTS

We initially sequenced complete *reg1α* gene in 50 patients and an equal number of controls and subsequently, additional patients and controls were screened for six SNPs with rare allele frequency of > 3%. Sequencing results revealed the presence of 8 reported SNPs, one novel SNP and one insertion-deletion polymorphism in the promoter region of the gene (Table 4). We did not observe any significant deviation from Hardy-Weinberg equilibrium ($P > 0.05$) for any of the polymorphisms. The polymorphisms in the promoter region were of prime interest, since the levels of *reg1α* expression differ considerably between the pancreas of patients and controls. Transplorer predicted 3 transcription factor-binding sites (C-Rel, -1513 to -1609; NFκB2, -1527 to -1614; and Hesx1, -15 to -105) within the region +10 to -1600 bp of the putative promoter region[21]. We sequenced the upstream region flanking the 5'-UTR (about 1176 bp upstream of translation start site) along with putative promoter region and found four reported SNPs, G-938A, T-912G, T-385C, T-243G which were equally frequent in patients and controls. A novel insertion-deletion polymorphism at -331 position (-331 to -329) involving

Table 4 Genotype data of polymorphisms analyzed in *reg1α* gene

Polymorphism	Patients (n = 195)			Controls (n = 150)		
	AA	Aa	aa	AA	Aa	aa
G-974C[1]	49	1	0	48	2	0
G-938A	92	73	30	74	52	24
T-912G	53	93	49	42	65	43
G-501A[1]	49	1	0	48	2	0
T-385C	98	76	21	80	52	18
-331delGGA	193	2	0	148	2	0
T-243G	92	73	30	69	58	23
G209T	125	61	9	103	43	4
G2199A	94	68	33	72	54	24
G2370A[1]	49	1	0	47	3	0

AA: Homozygous for major allele; Aa: Heterozygous; aa: Homozygous for minor allele. [1]Data from 50 patients & 50 controls.

Table 5 Haplotype frequencies of *reg1α* gene in patients with tropical calcific pancreatitis and healthy controls

S. No.	Haplotype	Haplotype frequency (%)		OR	P
		Patients (n = 195)	Controls (n = 150)	(95% CI)	value
1	GGTGGG	43.1	43.3	~1	~1
2	ATCTGA	30.3	31.3	0.95 (0.5-1.82)	0.88
3	GTTGTG	19.0	17.3	1.15 (0.52-2.5)	0.33
4	GGTTGG	2.1	2.0	~1	~1

OR: Odds ratio; 95% CI: 95% confidence interval; Haplotypes generated using six SNPs with minor allele frequency of > 3%, haplotypes with frequency > 2% are presented; Order of SNPs; G-938A, T-912G, T-385C, T-243G, G209T, G2199A in the reference sequence.

Table 6 Distribution of *reg1α* gene polymorphisms in tropical calcific pancreatitis patients based on N34S *SPINK1* and L26V *CTSB* status

SNP	*SPINK1* mutation				*CTSB* mutation			
	Minor allele frequency[1]		OR (95% CI)	P value	Minor allele frequency[2]		OR (95% CI)	P value
	N34S (n = 48)	WILD (n = 82)			L26V (n = 105)	WILD (n = 73)		
G-938A	0.33	0.34	0.96 (0.51-1.79)	0.88	0.34	0.31	1.15 (0.61-2.16)	0.65
T-912G	0.49	0.45	1.17 (0.65-2.13)	0.57	0.43	0.54	0.64 (0.35-1.17))	0.12
T-385C	0.31	0.33	0.91 (0.48-1.73)	0.76	0.31	0.26	1.28 (0.66-2.48)	0.43
T-243G	0.33	0.39	0.77 (0.41-1.43)	0.38	0.34	0.31	1.15 (0.61-2.16)	0.65
G209T	0.18	0.21	0.83 (0.39-1.76)	0.59	0.23	0.19	1.27 (0.61-2.66)	0.49
G2199A	0.34	0.35	0.96 (0.51-1.79)	0.88	0.38	0.27	1.66 (0.87-3.15)	0.10

SNP: Single nucleotide polymorphism; n: Number of individuals; OR: Odds ratio; CI: Confidence interval; [1]Minor allele frequency based on N34S *SPINK1* status; [2]Allele frequency based on L26V *CTSB* status.

deletion of GGA (-331delGGA) in the 5'UTR was identified but the frequency of deletion allele was similar in cases and controls. None of the seven polymorphisms in the promoter region altered the transcription-binding site and hence neither any existing transcription binding site was destroyed nor was a new site created. Other SNPs included two in the intronic region and one in the 3' UTR region of *reg1α* gene. All ten polymorphisms had comparable allele frequencies in patients and controls and the difference was statistically not significant (Table 2). Allelic odds ratio and confidence interval did not indicate an association with any of the polymorphisms identified in *reg1α* with TCP (Table 2). Haplotype analysis using the six *reg1α* polymorphisms with greater than 3% minor allele frequency supported the observations made from the allelic and genotypic data at different polymorphisms (Table 5). The patient population was divided into FCPD and TCP patients based on the presence or absence of diabetes, but we failed to observe any association between FCPD and polymorphisms in *reg1α* gene (Table 3). We also dichotomized the patient population based on the presence or absence of N34S mutation in the *SPINK1* gene and L26V mutation in the cathepsin B gene and compared the allele frequency of 6 SNPs in *reg1α* gene of patients having at least one mutant allele with those with the wild type pattern at the above mentioned mutations (Table 6), but could not detect any interaction between them and the *reg1α* variants.

DISCUSSION

TCP is associated with the presence of large calculi throughout the main pancreatic duct[25,26]. However, the mechanism of stone formation is not completely understood[26]. A decrease in tissular pancreatic stone protein mRNA concentration is associated with CCP[27,28]. The role of Reg proteins is debatable but they are known to be associated with pancreatic islet regeneration, diabetogenesis and amelioration of surgical diabetes in animal models[18]. Its role in pancreatic stone formation is not clear with suggestions that lithostathine could promote the nucleation of calcite crystals or may prevent pancreatic lithiasis by inhibiting calcite crystal nucleation and growth in the pancreatic juice[29]. Thus, mutations in *reg1α* gene could play an important role in the pathogenesis of TCP and FCPD.

A previous study, analyzed the exons of *reg1α* gene using a combination of Restriction fragment length polymorphism (RFLP), Single strand conformation polymorphism (SSCP) and sequencing techniques in 50 FCPD patients and controls, but did not identify any nucleotide substitutions and ruled out any contribution

of mutations in the coding regions of *reg1α* gene[30]. However, these workers did speculate about a possible role of regulatory variants in *reg1α* gene. A subsequent study also analyzed only the coding region in 12 Thai FCPD patients and 22 controls and ruled out any association with the disease[31]. T-385C, a polymorphism in exon 1 (5'UTR) with a moderately high allele frequency (0.32 in patients and 0.29 in controls) could have been missed in these studies due to the inherent limitations of techniques like SSCP in detecting any sequence changes. Our study involving extensive analysis of the gene as well as of the promoter region detected several polymorphisms including the promoter variants but the results suggest that there may not be any allelic or haplotypic association between the polymorphisms in *reg1α* and TCP.

As the *reg1α* gene is believed to be involved in islet cell repair and regeneration[18], we examined the association of *reg1α* variants with TCP and FCPD. The etiology and relationship of diabetes mellitus in FCPD are not well understood. Some believe that diabetes in FCPD is secondary to TCP while others suggest there is selective β-cell impairment, the latter hypothesis is supported by the occurrence of FCPD in some patients at a very young age. Evidence showing a preserved pancreatic α-cell function in diabetics with advanced chronic pancreatitis of the tropics indicates the presence of two different pathogenic mechanisms, one causing chronic pancreatitis and the other selective pancreatic β-cell impairment and subsequently diabetes mellitus[32]. However, an independent analysis of the TCP and FCPD patients did not suggest any role for *reg1α* variants in FCPD patients. Although, nearly one-half of the TCP patients carry N34S *SPINK1* mutation and the mutations in *SPINK1* and *CTSB* are the only genetic changes known to be associated with TCP, we did not find any evidence of an interaction between them. Although the present study had limited power to analyse such an interaction, our preliminary observations did not find a statistically significant difference in allele frequency between these groups for any polymorphism, suggesting the lack of epistatic interaction between *SPINK1* and/or *CTSB* with *reg1α* gene.

In conclusion, polymorphisms in *reg1α* gene, including those in the regulatory region are unlikely to contribute to the pathogenesis of pancreaticolithogenesis in tropical calcific pancreatitis. Other genes such as those involved in calcium signaling and regulation, either interacting with *reg* genes or functioning independently may play a role in stone formation in tropical calcific pancreatitis.

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COMMENTS

Background

Chronic pancreatitis (CP), an inflammatory condition of the pancreas with diverse etiologies, is usually associated with parenchymal calcification and presence of stones in the pancreatic duct. The process of stone formation in chronic pancreatitis is not completely understood. Lithostathine (encoded by *reg1α* gene), identified as a major proteic component of pancreatic stones in patients with alcoholic calcifying chronic pancreatitis, is thought to play an important role in the inhibition of stone formation and its levels are known to correlate with disease severity and is possibly regulated by the *reg1α* variants.

Research frontiers

Tropical calcific pancreatitis (TCP) and fibrocalculous pancreatic diabetes (FCPD; TCP presenting with diabetes) is a type of chronic pancreatitis specific to tropical countries. One of the important features of this condition is formation of large and irregular intraductal stones. Currently, there is considerable interest in understanding the mechanism of stone formation, the factors that inhibit stones, the genes involved in the process of pancreaticolithiasis as well as the effect of various polymorphisms. An additional area of interest is the relationship between the pancreatic inflammation and pancreaticolithiasis as well as the influence of genetic variants that predict susceptibility to the development of chronic pancreatitis.

Innovations and breakthroughs

The present study attempted to open new frontiers in the area of molecular pathogenesis of stone formation in TCP and FCPD by ruling out the role of *reg1α* variants in pancreaticolithiasis.

Applications

The results of the present study propose a new assessment of the pathogenesis of stone formation in TCP and FCPD. Further studies should be designed to elucidate more information.

Terminology

The process of stone formation, lithogenesis, is believed to be initiated by calcite nucleation with the subsequent deposition of proteins leading to protein plug formation; Lithostathine C is known to influence this process.

Peer review

The authors of this manuscript screened the *reg1α* gene including the regulatory region by sequencing and examining the association of the polymorphisms in the gene with pancreaticolithiasis in TCP and FCPD. The authors conclude that neither the previously reported nor novel variants in the *reg1α* gene predict the susceptibility to pancreaticolithiasis in TCP and FCPD.

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RAPID COMMUNICATION

In-vitro activation of cytotoxic T lymphocytes by fusion of mouse hepatocellular carcinoma cells and lymphotactin gene-modified dendritic cells

Xi-Ling Sheng, Hao Zhang

Xi-Ling Sheng, Department of Intensive Care Unit, the Red Cross Hospital, Hangzhou 310003, Zhejiang Province, China
Hao Zhang, Department of Hepatobiliary Surgery, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
Supported by the Science & Technology Foundation for Academicians of Zhejiang Province, China, No. 203201513
Correspondence to: Dr. Hao Zhang, Department of Hepatobiliary Surgery, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China. zhanghao111111@tom.com
Telephone: +86-571-87236570 Fax: +86-571-87236570
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Abstract

AIM: To investigate the *in-vitro* activation of cytotoxic T lymphocytes (CTLs) by fusion of mouse hepatocellular carcinoma (HCC) cells and lymphotactin gene-modified dendritic cells (DCs).

METHODS: Lymphotactin gene modified DCs (DCLptn) were prepared by lymphotactin recombinant adenovirus transduction of mature DCs which differentiated from mouse bone marrow cells by stimulation with granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and tumor necrosis factor alpha (TNF-α). DCLptn and H22 fusion was prepared using 50% PEG. Lymphotactin gene and protein expression levels were measured by RT-PCR and ELISA, respectively. Lymphotactin chemotactic responses were examined by *in-vitro* chemotaxis assay. *In-vitro* activation of CTLs by DCLptn/H22 fusion was measured by detecting CD25 expression and cytokine production after autologous T cell stimulation. Cytotoxic function of activated T lymphocytes stimulated with DCLptn/H22 cells was determined by LDH cytotoxicity assay.

RESULTS: Lymphotactin gene could be efficiently transduced to DCs by adenovirus vector and showed an effective biological activity. After fusion, the hybrid DCLptn/H22 cells acquired the phenotypes of both DCLptn and H22 cells. In T cell proliferation assay, flow cytometry showed a very high CD25 expression, and cytokine release assay showed a significantly higher concentration of IFN-γ and IL-2 in DCLptn/H22 group than in DCLptn, DCLptn+H22, DC/H22 or H22 groups. Cytotoxicity assay revealed that T cells derived from DCLptn/H22 group had much higher anti-tumor activity than those derived from DCLptn, H22, DCLptn+H22, DC/H22 groups.

CONCLUSION: Lymphotactin gene-modified dendritoma induces T-cell proliferation and strong CTL reaction against allogenic HCC cells. Immunization-engineered fusion hybrid vaccine is an attractive strategy in prevention and treatment of HCC metastases.

Key words: Hepatocellular carcinoma; Dendritic cell; Cytotoxic T lymphocyte

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INTRODUCTION

Dendritic cells (DCs) are the most important antigen-presenting cells (APCs)[1-3]. DCs-based vaccinations have been demonstrated to be effective in inducing antigen-specific cytotoxic T lymphocyte (CTL) responses[4-9]. Previous studies in mouse tumor models or cancer patients demonstrated that vaccination with hybridomas from tumor cells and DCs induces regression of established carcinomas, lymphomas and myeloma[10-16]. This study was to investigate the *in-vitro* immune effects of fusion of mouse hepatocellular carcinoma (HCC) cells and lymphotactin (Lptn) gene-modified DCs and its antitumor activity.

MATERIALS AND METHODS

Animals, recombinant adenoviruses and cell lines

Five- to six-week old Female BALB/c (H-2K^d) mice were obtained from the Animal Resource Center of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, and maintained in specific pathogen-free conditions for use at the age of 6-8 wk. Recombinant Ad5 adenoviruses harbouring mouse lymphotactin (AdLptn) or LaZ gene (AdLacZ) were kindly provided by Dr. Cao Xue-Tao. The recombinant adenoviruses were propagated in

human embryonic kidney 293 (HEK293) cells, and purified by cesium chloride (CsCL) density gradient centrifugation. Titers of AdLptn and AdLacZ determined by plaque assay on HEK293 cells were 3.6×10^9 plaque-forming units (PFU)/mL and 4.5×10^9 PFU/mL, respectively. H22 cells, established as a BALB/c mouse origin HCC cell line, were purchased from China Center for Type Culture Collection. All the cells were cultured in RPMI-1640 (H22 cells) or DMEM (HEK293 cells) medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

DC culture

DCs were prepared as previously described[17] with certain modifications. Briefly, bone marrow cells prepared from femora and tibias of normal BALB/c mice were depleted of red blood cells with ammonium chloride and plated in RPMI-1640 plus 10% FCS and 10 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF; R&D) with conjunction of 10 ng/mL interleukin-4 (IL-4; R&D) on d 1. On d 3, nonadherent granulocytes, T and B cells were gently removed and fresh media were added. On d 5, loosely adherent proliferating DC aggregates were dislodged and re-plated in the fresh media, and supplemented with 50 ng/mL tumor necrosis factor-α (TNF-α; R&D). On d 7, the released nonadherent mature DCs were harvested. CD11c-positive DCs accounted for more than 80% of the harvested cells as measured by flow cytometry.

Adenovirus transduction

Cultured DCs were pelleted and washed with PBS prior to the addition of virus. Virus stock (stored at -80℃) was thawed at room temperature and diluted in serum-free RPMI-1640 medium. The pellets of DCs were resuspended in serum-free RPMI-1640 and virus was added. After 2 h incubation with virus, cells were washed once in PBS. DCs were resuspended in a cytokine-supplemented medium which was retained after DC culture. Twenty-four hours after gene modification, LacZ gene-modified DCs (DCLacZ) were collected for X-gal staining to evaluate the gene transfer efficiency. Lymphotactin gene-modified DCs (DCLptn) were collected for phenotypic analysis and fused with H22 cells *in vitro*.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from cells using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. cDNA was prepared from total RNA using a hexanucleotide random primer and SuperScrip Moloney murine leukemia virus reverse transcriptase (Life Technologies). PCR primers for the amplification of mouse lymphotactin and beta-actin used are as follows (lymphotactin forward primer: 5'TGGG GACTGAAGTCCTAGAAG3'; reverse primer: 5'TTA CCCAGTCAGGGTTACTGCTGCTGTG3', with the product size of 300 bp. Beta-actin forward primer: 5'TG GAATCCTGTGGCATCCATGAAAC3'; reverse primer: 5'TAAAAGCCAGCTCAGTAACAGTCCG3', with the expected size of 359 bp.). PCR was performed in a Perkin Elmer Cetus DNA thermal cycler using Taq DNA polymerase (Life Technologies). The program consisted of 25 cycles of template denaturation at 94℃ for 1 min, annealing of primers at 60℃ for 1 min and synthesis at 72℃ for 2 min, followed by a final extension at 72℃ for 10 min. The PCR products were analyzed by agarose gel electrophoresis. Controls without reverse transcriptase were used to confirm that the RT-PCR products obtained were not the result of contamination with genomic DNA.

ELISA for measuring lymphotactin in supernatants

Lymphotactin protein in the supernatants from DCLptn was quantitatively determined with a commercial "sandwich" enzyme immunoassay kit (R&D) according to the manufacture's instructions. Briefly, Costar EIA microplates were coated with 100 μL of 2 μg/mL rat-anti-mouse lymphotactin as a capture antibody, incubated overnight at room temperature, and blocked with 1% bovine serum albumin (BSA) in PBS. Then, 100 μL of serially diluted standards or culture supernatant samples was added in triplicate and incubated at room temperature for 2 h. The plates were washed and incubated at room temperature for 2 h with 100 μL of 400 ng/mL biotinylated goat anti-mouse lymphotactin as a detection antibody. After washing, wells were incubated for 20 min in 100 μL of streptavidin- horseradish peroxidase (HRP) solution, and developed with substrate solution.

Cell fusion

DCLptn were fused with tumor cells at a 3:1 (DC: tumor) ratio using 50% polyethylene glycol (PEG, 50% PEG/10% DMSO in PBS, Sigma). In brief, H22 cells were inactivated by 30 μg/mL mitomycin, washed and mixed with DCLptn. After centrifugation, 1 mL of 50% PEG was added to the cell pellets for 2 min at 37℃. Then, an additional 10 mL of warm serum-free medium was added to dilute PEG over the next 3 min with continuous stirring. PEG-treated cells were centrifuged at 400 × *g* for 5 min, resuspended with RPMI-1640 medium supplemented with 20% FCS, 10 ng/mL GM-CSF and 10 ng/mL IL-4, and cultured overnight.

To determine the efficiency of cell fusion, H22 cells were stained with PKH-26 (red fluorescence, Sigma) and DCLptn were stained with PKH-2 (green fluorescence, Sigma). The cells stained with the fluorescence dyes were treated with PEG and cultured overnight as described above. On the next day, the stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson) under a confocal microscope.

Phenotypic analysis

After washing, cells were resuspended in PBS containing 1% BSA, and stained with fluorescence-conjugated monoclonal antibody (H-$2K^d$, I-A^d, CD80, CD86, CD40, CD54) or isotype control antibody for 30 min at 4℃. The stained cells were washed and analyzed using FACS

In vitro chemotaxis assay

Chemotactic responses of lymphotactin to T cells were examined using modified boyden microchemotaxis chambers (Neuro Probe, Gaithersburg) and polyvinyl pyrrolidone-free 5 μm pore size polycarbonate

membranesy. Briefly, spleen cells from naïve BALB/C mice were used as effector cells. The bottom wells of the chamber were loaded with supernants of H22, DC, DCLptn, DCLacZ or RPMI-1640 alone, and the upper wells contained 1×10^5 effector cells. After 1 h incubation and staining, data were obtained by counting five nonoverlapping high power microscopic fields from each well. Cells were considered chemoattracted if the chemotactic index (number of cells migrating in experimental well/number of cells migrating in RPMI-1640 medium only) was greater than 2.

CD25 expression and cytokine production after autologous T cell stimulation

To determine the proliferation and differentiation of lymphocytes, CD25 expression and cytokine production after autologous T cell stimulation were assayed. Briefly, spleen cells from naïve BALB/C mice were passed over nylon wool with their purity determined by FACS (percentage of $CD3^+$ cells near 90%) and used as responder cells at 1×10^5/well in 96-well U-bottom plates. Syngeneic H22, DCLptn, H22+ DCLptn (H22 cells co-cultured with DCLptn at a ratio of 3:1), DC/H22 (H22 cells fused with DC at a ratio of 3:1) and DCLptn/H22 (H22 cells fused with DCLptn at a ratio of 3:1) cells were inactivated with 30 μg/mL mitomycin for 30 min and added to responder cells in varying cell numbers. Cells were cultured at 37℃ in RPMI-1640 medium containing 10% FCS and 5% CO_2 for 2 d. Control wells contained T cells alone. At the end of experiment, supernatants were harvested for cytokine production assay by ELISA and co-cultured T-cells were collected for analyzing CD25 expression by FACS.

CTL assay

Cytotoxic function of the activated T lymphocytes stimulated with DCLptn/H22 was determined by cytotoxicity test. Inactivated cells were co-cultured with spleen T cells separated from naïve BALB/C mice at a 1:10 ratio in the presence of 20 U/mL mouse IL-2 for 7 d. The stimulated T cells were isolated and used as effector cells in lactate dehydrogenase (LDH, Roche) cytotoxicity assay. H22 cells were used as target cells. All steps were performed following the manufacturer's instructions. Briefly, after washed with assay medium (RPMI1640 with 1%BSA), the effector cells were co-cultured at 37℃ with target cells in a 96-well round bottom plate for 6 h, then the plate was centrifuged and the supernatants were transferred to another flat-bottom ELISA plate. One hundred μL of LDH detection mixture was added to each well and incubated at room temperature in the dark for 30 min. Absorbance was measured with an ELISA reader at 490 nm. The spontaneous release of LDH by target cells or effector cells was assayed by incubation of target cells in the absence of effector cells and vice versa, the maximum release of LDH was determined by incubation of the target cells in 1% Triton X-100 in assay medium. The percentage of cell-mediated cytotoxicity was determined by the following equation: cytotoxicity (%) = [(mixture of effectors and targets-effector control)/(maximum-spontaneous)] × 100.

Statistical analysis

Data were expressed as mean ± SD. Experiment results were analyzed using SPSS 10.0 statistical package. Differences among groups were assessed by the Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Lymphotactin expression and functional assay

DC and H22 did not express any detectable Lptn, which was detected in DCLptn and H22Lptn (Figure 1). The results indicate that adenovirus vector could effectively transducer the Lptn gene.

In order to quantitatively determine Lptn protein in supernatants from gene-modified DCs, culture supernatants were harvested and determined for Lptn production by ELISA. The results showed that about 0.35 ± 0.04 ng/mL Lptn could be detected in the supernatants of DCLptn, while nearly no Lptn could be detected in the supernatants from untransfected DC, DCLacZ and H22 cells.

Consistent with ELISA results, only the supernatant from DCLptn was positive for chemotaxis assay (chemotaxis index = 3.2 ± 0.15), but from DC, DCLacZ, H22 groups was negative. The results indicate that recombinant Lptn secreted from DCLptn had an effective biological activity.

Recognition and characterization of H22 and DCLptn fusion

Fusion was examined by confocal microscopy (Figure 2) and flow cytometry (Figure 3). The fusion cells were yellow under confocal microscope. The fusion efficiency assayed by FACS was 15%-22%.

FACS analysis showed that DCs encoding lymphotactin were positive for H2-K^d, I-A^d, CD80, CD86, CD40, CD54. However, H22 cells expressed a moderate level of I-Ad. The expression levels of H-2K^d, CD80, CD86, CD40 and CD54 were almost negative. Hybrid DCLptn/H22 cells acquired the phenotypes of both DCLptn and H22 cells.

Enhancement of Th1 cytokine production and CD25 expression

Flow cytometry showed that a very high CD25 expression was observed in T lymphocytes generated in autologous mixed lymphocyte reaction with DCLptn/H22 fusions (58.23% ± 11.65%) when compared to T cells either cultured with DCLptn cells (39.12% ± 12. 35%), H22 (10.78% ± 5. 46%), DC/H22 cells (41.55% ± 12.82%), or DCLptn+H22 cells (43.03% ± 10.52%). By *in vitro* cytokine release assay, significantly higher concentrations of IFN-γ and IL-2 were noted in supernatants of DCLptn/H22 co-cultured with T cells compared to those of DCLptn, DCLptn + H22, DC/H22 or H22 co-cultured with T cells. No difference was noted between concentrations of IL-4 or IL-10 in supernatants of all groups (Table 1).

Elicitation of tumor-reactive CTLs by fusion of DCs with H22 cells

Cytotoxic assay revealed that T cells derived from

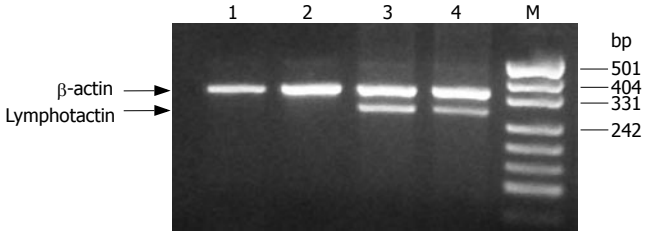

Figure 1 RT-PCR analysis of lymphotactin gene expression in DC, H22, DCLptn, H22Lptn (lanes 1-4). The data shown are representative of three separate analyses for each cell population. M: Marker.

DCLptn/H22 group possessed an extremely higher anti-tumor activity than those derived from DCLptn, H22, DCLptn + H22, DC/H22 groups. Although there were no differences among DCLptn, DCLptn + H22 and DC/H22 groups, the anti-tumor activity of DCLptn, DCLptn + H22 and DC/H22 groups was remarkably higher than that of H22 groups (Figure 4).

DISCUSSION

$CD8^+$ T cells are critical components in immune responses to tumors and can differentiate into cytotoxic T lymphocytes and acquire the ability to lyse tumor antigen expressing cells. Activation of $CD8^+$ T cells requires two steps[18-20]: presentation of antigenic peptides on professional antigen presenting cells and helper function provided by $CD4^+$ T cells *via* Th1/Th2 cytokines. When DCs and HCC cells are fused, antigens are processed and displayed on the cell surface through MHC class I pathway which stimulates $CD8^+$ T cells, and some antigens may be displayed by MHC class Ⅱ molecules, which stimulate $CD4^+$ T cells. On the other hand, mature DCs express MHC Ⅰ, MHC Ⅱ and co-stimulatory molecules that provide necessary signals for the stimulation of naïve T cells[21,22]. Upon stimulation, proliferating $CD4^+$ T lymphocytes differentiate along the Th1 pathway, resulting in increased IFN-γ and IL-2 production, contributing to the activation of tumor-specific CTLs and enhancing the cytotoxic effect. Evidence from cytokine release assays indicates that in cultures with proliferating lymphocytes, the production and secretion of Th1-associated cytokines (IFN-γ, IL-2) but not Th2-associated cytokines (IL-4, IL-10) are increased. In our study, the fusion groups had a higher CTL activity than H22 group.

Activation of lymphocytes is a dynamic, multistep process. Although MHC and costimulatory molecules are critical for successful T-cell activation, signals that regulate this process have not been fully elucidated. It is believed that chemokines are an essential mediator. Migration of DCs to the sites of inflammation where they capture antigens and subsequently migrate to the local lymph nodes is regulated by the expression of different chemokines and their receptors[23,24]. Lymphotactin as a C chemokine produced mainly by T and nature killer (NK) cells, is a chemoattractant both *in vitro* and *in vivo*[25-28]. In our study, DCs and H22 cells did not express Lptn, and the Lptn gene-modified hybridima had a stronger CTL activity

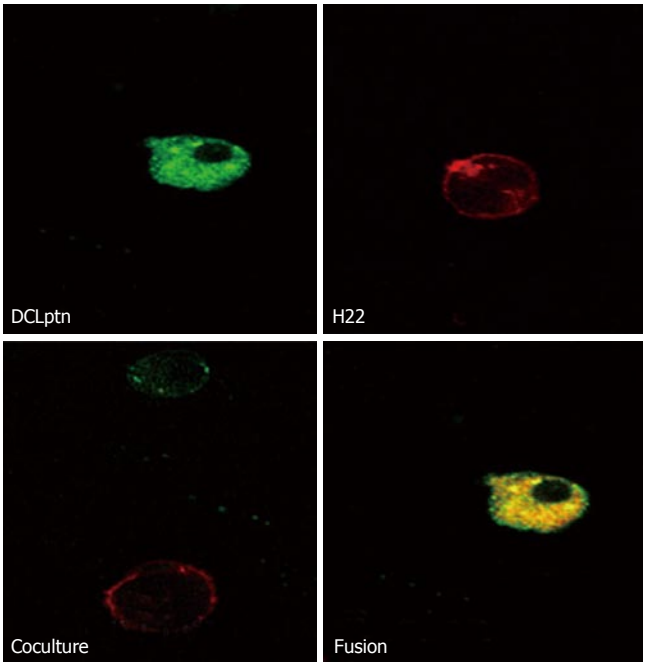

Figure 2 Confocal micrography of DCLptn/H22 fusion cells.

and a higher Th1 cytokine production, suggesting that Lptn modification can improve preferential chemotaxis of hybridoma on T cells and consequently optimize the microenvironment of antigen presentation to T cells.

CD25, α-chain of the IL-2 receptor, is expressed in the early to moderate phase after T-cell activation, the clonal proliferation of activated T cells depends on the expression of this receptor and resting lymphocytes do not express CD25[29,30]. Therefore, CD25 expression is commonly used as a marker for T cell activation. Quantification of surface IL-2 receptor expression on activated lymphocytes by flow cytometry after *in vitro* stimulation with specific antigens is useful in measuring cellular immunity. In the present study, we used this method to assess the lymphotactin gene-modified hybridoma's stimulation on co-cultured T cells. By using this method, we were able to study the effect of stimulation on a heterogeneous cell population without the risk of selective depletion of cells, to exclude non-specific stimulation due to the separation, and to express CD25 at the early to moderate (24-48 h) phase of mixed lymphocyte reaction, thus shortening the co-culture time and keeping the viability of T cells.

In conclusion, lymphotactin gene-modified dendritoma induces potent T-cell proliferation and strong CTL reaction against allogenic HCC cells. Immunization-engineered fusion hybrid vaccine is an attractive strategy in prevention and treatment of cancer metastases.

COMMENTS

Background

Despite recent advances in surgical technique and radio- and chemotherapy, the prognosis of patients with malignant tumors remains dismal. The resistance of these tumors to conventional treatment may stem from their well-documented ability to exert local and systemic immunosuppressive effects. Therefore, alternative treatments are required.

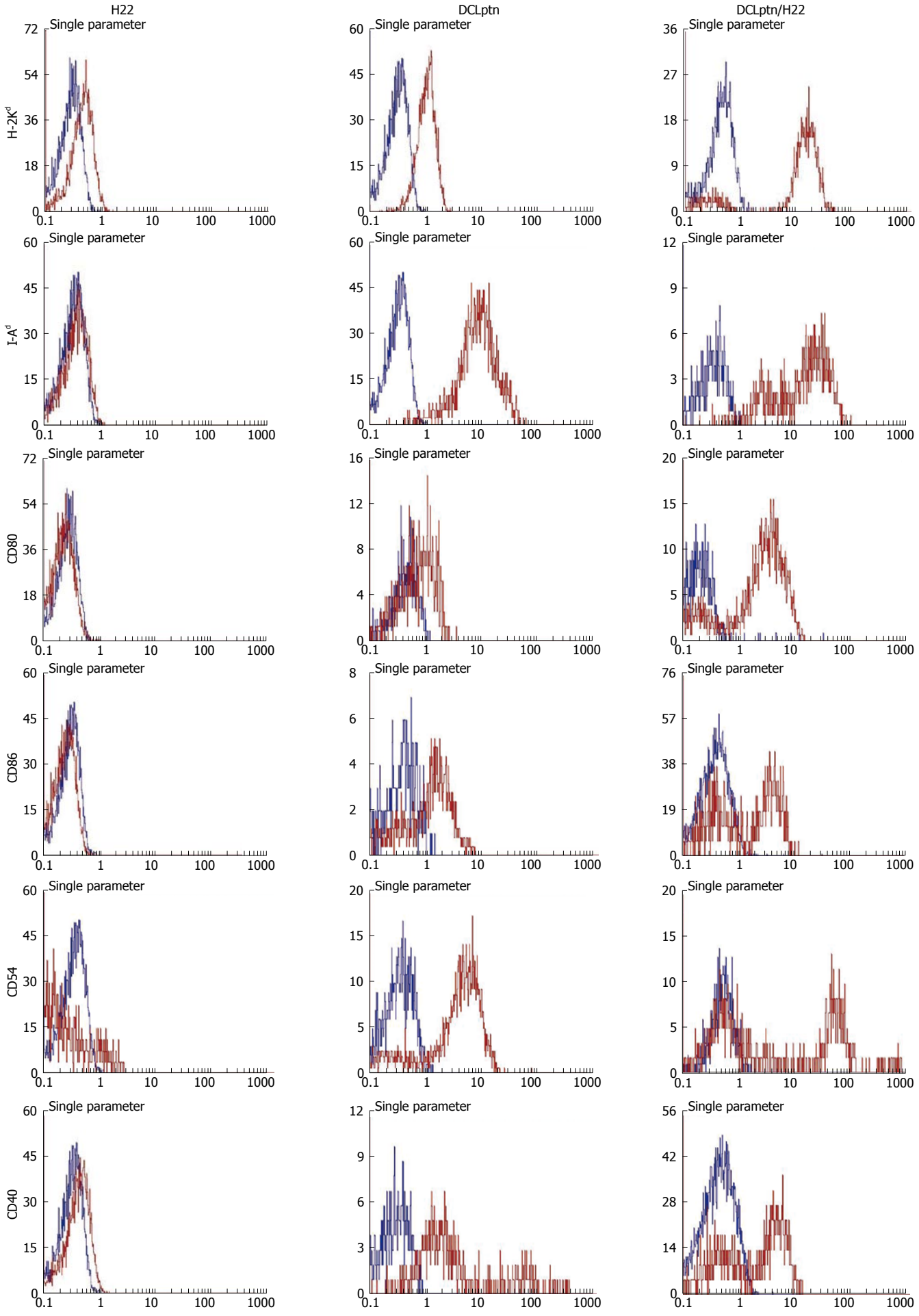

Figure 3 FACS analysis of the phenotypes of H22, DCLptn and DCLptn/H22 fusion cells.

Table 1 Cytokine concentration in MLR supernatants after different cell population stimulation (pg/mL, mean ± SD)

	IFN-γ	IL-2	IL-10	IL-4
A (H22)	510.3 ± 9.32	39.7 ± 2.72	91.48 ± 1.59	58.64 ± 0.4
B (DCLptn)	1015.51 ± 7.2[f]	88.47 ± 3.17[f]	96.20 ± 1.27	55.89 ± 2.95
C (DCLptn+H22)	999.64 ± 11.86[f]	82.39 ± 3.02[f]	97.33 ± 2.23	59.78 ± 1.21
D (DC/H22)	992.45 ± 10.16[d,f]	93.28 ± 0.91[d,f]	131.94 ± 0.32[d]	98.71 ± 2.14[d]
E (DCLptn/H22)	1886.08 ± 56.75[b]	170.12 ± 2.11[b]	217.13 ± 1.91[b]	167.58 ± 0.94[b]

[b]$P < 0.01$ *vs* A, B, C, D; [d]$P < 0.01$ *vs* A, B, C; [f]$P < 0.01$ *vs* A.

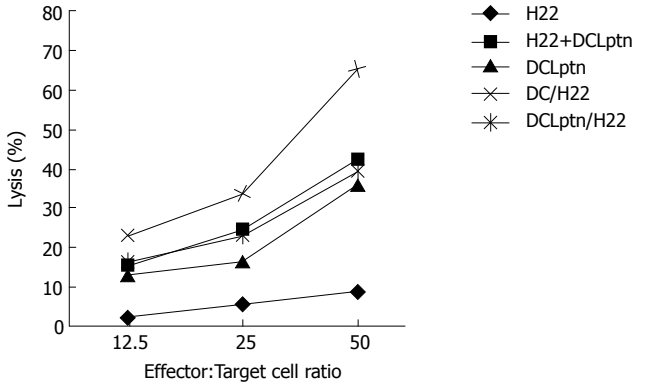

Figure 4 Stimulation of anti-tumor CTLs by DCLptn/H22 cells.

Research frontiers

Dendritic cells are the most potent APC for inducing an antigen-specific CTL response. This property, coupled with the fact that it is now possible to generate, *ex vivo*, a large number of functional dendritic cells from a patient's peripheral blood monocytes or CD34 haemopoietic stem cells, have led to a considerable interest in use of dendritic cell vaccines as a means to induce antitumour immunity. Various strategies have been developed to introduce tumor specific antigens into DCs and thereby to generate cytotoxic T lymphocyte (CTL) responses against malignant cells. One of the important approaches to the induction of primary antitumor immunity is through the generation of tumor cell and DC fusion.

Innovations and breakthrough

Although some effective results have been obtained by vaccinating mice with fusion of DCs and other tumor-cell types, it still remains a challenge. Several parameters must be optimized in order to maximize the efficacy of immunotherapy for dendritoma. In the present study, the authors have found that after Lptn gene modification, activated T cells can acquire more tumor antigens from DCLptn/H22 and have a stronger cytotoxicity to target cells.

Applications

This may be an attractive strategy in prevention and treatment of cancer metastases.

Terminology

Dendritoma: fusion formed by dendritic cells and carcinoma cells.

Peer review

This paper investigated the *in vitro* activation of cytotoxic T lymphocytes by fusion of mouse hepatocellular carcinoma (HCC) cells and dendritic cells modified by transfection of the lymphotactin gene. The authors conclude that lymphotactin modifies dendritoma and induces T cell proliferation and strong reaction of cytotoxic lymphocytes against allogenic HCC cells. These results are of certain interest.

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CASE REPORT

Poorly differentiated carcinoma of the rectum with aberrant immunophenotype: A case report

A Giannopoulos, I Papaconstantinou, P Alexandrou, A Petrou, A Papalambros, E Felekouras, E Papalambros

A Giannopoulos, I Papaconstantinou, P Alexandrou, A Petrou, A Papalambros, E Felekouras, E Papalambros, First Department of Surgery, Athens Medical School, National and Kapodistrian University of Athens, LAIKO General Hospital, Greece
Correspondence to: Dr. Ioannis Papaconstantinou, SpR in General Surgery, First Department of Surgery, National and Kapodistrian University of Athens Medical School, LAIKO Hospital, 17 St. Thomas St, Athens 11527, Greece. johnpapacon@yahoo.com
Telephone: +30-69-32906329
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Abstract

We report a case of a poorly differentiated epithelial tumour of the rectum with a highly pleomorphic morphology and an aberrant immunophenotype, including the expression of epithelial markers, the focal parameter of neuroendocrine differentiation, and the unexpected detection of CD-117 overexpression. A 69-year-old man was admitted to our clinic complaining of rectal bleeding and weight loss. Colonoscopy showed an ulcerative bleeding mass located about 8 cm from the anal verge. Abdominal and pelvis CT scans demonstrated a large low-density lesion with extracanalicular growth from the middle rectum, with local lymph-node spread, and without tumour infiltration of other pelvic organs, or evidence of distant intra-abdominal spread. The patient underwent a low anterior resection for rectal cancer together with wide resection of lymph nodes. In immunohistochemical analysis, pankeratin and Epithelial Membrane Antigen (EMA) immunolabeling proved the epithelial nature of the tumor cells. Chromogranin A and Leukocyte Common Antigen (LCA) were negative, whereas CD-56 expression was scanty and Neuron Specific Enolase (NSA) was heavily and diffusely expressed. Ki67 immunoexpression was particularly increased. Interestingly, the intense c-kit immunoreactivity (100%) was a common feature. The above phenotypic and immunohistochemical profile was consistent with an anaplastic carcinoma of the large intestine, with focal neuroendocrine differentiation and diffuse immunoreactivity to c-kit protein. Given the resistance of this tumor to conventional chemotherapy and radiation, the incidence of the c-kit alteration may represent a novel approach to a gene-directed treatment using a c-kit inhibitor (STI571) similar to that which has been proposed in GISTs.

INTRODUCTION

We report a case of a poorly differentiated epithelial tumour of the rectum with a highly pleomorphic morphology and an aberrant immunophenotype, including the expression of epithelial markers, the focal parameter of neuroendocrine differentiation, and the unexpected detection of CD-117 overexpression.

CASE REPORT

A 69-year-old man was admitted to our clinic complaining of rectal bleeding for 2 mo (two episodes of massive rectal bleeding) and weight loss of 5 kg in 4 mo. His past medical history was negative for any surgical procedure or chronic disease, and his family history was also free. He denied any change in bowel habits, urinary urgency, or any other symptoms. Digital examination was normal but proctosigmoidoscopy showed an ulcerative mass bulging over the right rectal wall, and the fecal examination was positive for blood.

Laboratory tests of the peripheral blood revealed microcytic hypochromic anemia (hemoglobin, 11.7 g/dL and hematocrit, 26.6%). The serum levels of carcinoembrionic antigen (CEA), alpha-fetoprotein AFP, and CA19-9 were within normal ranges. Prostate-specific antigen (PSA) was also within the normal range (0.7 ng/dL, PSA free, 0.16 mg/dL).

Colonoscopy showed an ulcerative bleeding mass that was located about 8 cm from the anal verge. An additional abnormality revealed by colonoscopy was the existence of five small polyps along the rest of the colon. Abdominal and pelvis CT scans demonstrated a large low-density lesion with extracanalicular growth from the middle

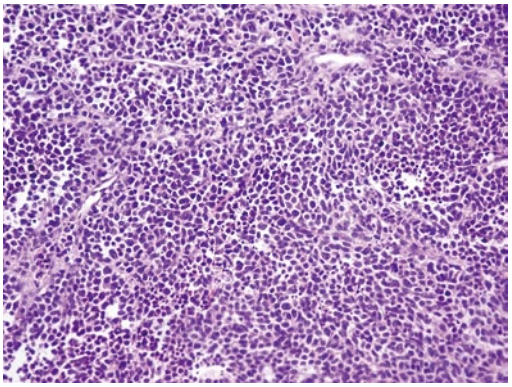

Figure 1 Histological appearance of the colorectal adenocarcinoma (HE, × 20).

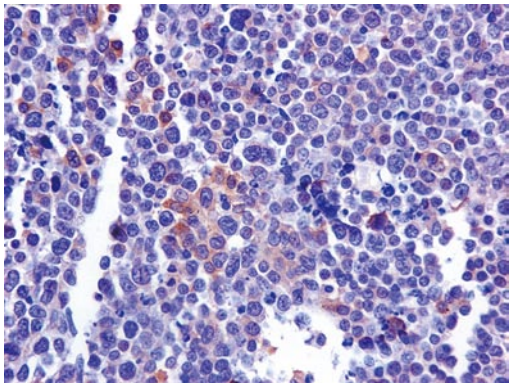

Figure 2 Scanty CD-56 immunohistochemical expression by tumor cells (× 40).

rectum, with local lymph-node spread, and without tumour infiltration of other pelvic organs, or evidence of distant intra-abdominal spread. No metastatic nodules were found in the lung and the liver by diagnostic imaging procedures. The patient underwent a low anterior resection for rectal cancer with a circular stapled low, end-to-end colorectal anastomosis (indicated for tumours situated 6-9 cm above the anal verge), together with wide resection of lymph nodes.

On gross examination, the 14-cm rectosigmoidal surgical specimen manifested as an ulcerative tumor that measured 5 cm in its larger diameter, located 2-5 cm from the distal resection margin.

Under microscopy, the tumor was composed of irregular sheets and scattered tumor cells (Figure 1) with markedly pleomorphic nuclei and prominent nuclei, including giant or multinucleated cell types. The tumor was found to infiltrate the submucosa, the muscularis propria, and the perirectal adipose tissue. Nodal metastasis was found in 2/22 lymph nodes examined.

In immunohistochemical analysis, pankeratin and Epithelial Membrane Antigen (EMA) immunolabeling proved the epithelial nature of the tumor cells. Chromogranin A and Leukocyte Common Antigen (LCA) were negative, whereas CD-56 expression was scanty (Figure 2), and Neuron Specific Enolase (NSA) was heavily and diffusely expressed. Ki67 immunoexpression was particularly increased. Interestingly, the intense c-kit immunoreactivity (100%) was a common feature (Figure 3A and B). The above phenotypic and immunohistochemical profile was consistent with an anaplastic carcinoma of the large intestine, with focal neuroendocrine differentiation and diffuse immunoreactivity to c-kit protein.

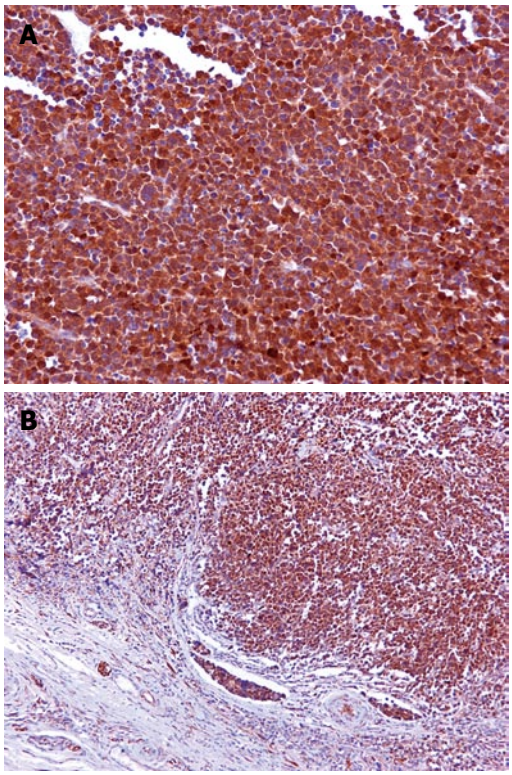

Figure 3 **A**: Intense c-kit immunolabeling (× 20); **B**: intense nuclear cytoplasmic immunolabeling for c-kit protein (× 10).

DISCUSSION

c-kit protein, a 145-kDa tyrosine kinase with oncogenic properties is a transmembrane receptor growth factor known as a stem cell factor (SCF). It is encoded by the c-kit proto-oncogene located on chromosome 4q11-q12[1]. Activation of c-kit by its SCF ligand leads to dimerization of the receptor. The latter activates further signalling cascades that control cell proliferation, adhesion and differentiation[2].

CD-117 is a functionally important protein in hematopoietic stem cells, mast cells, germ cells, some epithelial cells and in Cajal cells. Parenthetically, Cajal cells are known to originate from common intestinal mesenchymal precursor cells[2-5].

Several studies have identified the presence of a c-kit malignant mutation in over half of gastrointestinal stromal tumors (GISTs), as well as in other human tumors, including germ cell tumors, neuroblastoma, melanoma, ovarian carcinoma and breast carcinoma[6-14]. Interestingly, overexpression of c-kit has been found to affect proliferation in human neural, lung, breast, colorectal, skin and prostatic tumors[15].

On the basis of an immunohistochemical study of c-kit expression in 126 colorectal carcinomas, only two

(1.6%) poorly differentiated carcinomas presented with aberrant c-kit positivity, which implies the role of c-kit in tumor progression[16]. Although the functional role of mutated c-kit kinase activity is not fully understood, it seems that in breast, thyroid and ovarian cancer, the malignant transformation seems to correlate with loss of c-kit protein expression[17]. However, Bellon *et al*[12] have reported overexpression of c-kit in human colorectal cancer, and have suggested that c-kit activation is critical for growth, survival, migration and invasive potentional of DLD-1 colon carcinoma cells. Of interest, only 1.6% of colorectal cancers show high cytoplasmic c-kit staining, a fact that is not related definitely to tumorigenesis[16]. Immunohistochemical expression of c-kit protein is a rare event in poorly differentiated carcinomas[16,17].

In the study by Akintola-Ogunremi *et al*[17], who studied 66 cases of primary colorectal neuroendocrine carcinoma, the prognosis did not appear to differ between kit-positive and kit-negative cases. In the view of the limited number of reports in the literature and the lack of follow-up data, c-kit overexpression cannot provide any evidence regarding the biological behavior of the tumor currently described. However, further follow-up, together with c-kit gene mutational analysis may alter the prognostic value of c-kit positivity in these highly aggressive malignancies of the colon. Thus, the immunohistochemical CD-117 alteration in poorly differentiated carcinoma of the rectum remains to be elucidated.

Given the resistance of this tumor to conventional chemotherapy and radiation[18,19], the incidence of the c-kit alteration may represent a novel approach to a gene-directed treatment using a c-kit inhibitor (STI571) similar to that which has been proposed in GISTs[20]. According to the literature, STI571 may inhibit the *in vitro* growth of colorectal carcinoma cell lines, although it has not been tested so far for the treatment of colorectal carcinoma[20].

A long term study of c-kit protein expression in poorly differentiated malignancies of colon may be warranted, although c-kit overexpression can not guarantee tumor response. Thus, a thorough genetic investigation of colorectal malignancy may determine the eligibility of STI571 regimen for potential targeted therapy.

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CASE REPORT

Antegrade bowel intussusception after remote Whipple and Puestow procedures for treatment of pancreas divisum

Manuel Gigena, Hugo V Villar, Negar G Knowles, John T Cunningham, Erik K Outwater, Luis R Leon Jr

Manuel Gigena, Hugo V Villar, Negar G Knowles, John T Cunningham, Erik K Outwater, Luis R Leon Jr, University of Arizona Health Science Center, and Southern Arizona Veteran Affairs Health Care System, Surgery Section, Tucson, AZ 85723, United States
Correspondence to: Luis R Leon Jr, MD, RVT, University of Arizona Health Science Center, and Southern Arizona Veteran Affairs Health Care System, Surgery Section, 3601 South 6th Avenue, Vascular Surgery Section Room N259, Tucson, AZ 85723, Arizona, United States. luis.leon@va.gov
Telephone: +1-520-7921450-16596
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Abstract

To date, antegrade intussusception involving a Roux-en-Y reconstruction has been reported only once. We report a case of acute bowel obstruction due to an intussusception involving two Roux-en-Y limbs in a 40-year-old woman with a history of chronic pancreatitis due to pancreas divisum. Four years preceding this event, the patient had undergone a Whipple procedure, and three years prior to that, a Puestow operation. The patient was successfully treated with bowel resection and a side-to-side anastomosis between the most distal aspect of the bowel and the most distal Roux-en-Y reconstruction, which preserved both Roux-en-Y reconstructions.

Key words: Whipple procedure; Puestow procedure; Pancreas divisum; Intussusception; Bowel obstruction

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INTRODUCTION

It is a well known fact that intussusception is most often seen in children[1]. Intussusception in adults however is relatively rare, with about 17% of intussusception cases in large reported series having occurred in adults[1]. Surgical sutures or staples along an anastomosis are, among other factors, well-known lead points for the development of intussusception, therefore making abdominal surgical interventions recognized risk factors for the occurrence of this complication[2-6]. Intestinal tract reconstructive surgery involving the pancreas however, has been very rarely linked to the development of intussusception[7]. We report the case of a middle-aged woman who developed intussusception after two major operations that were remotely performed for the therapy of symptomatic pancreas divisum. A brief discussion of the available literature is also presented.

CASE REPORT

A 40-year-old woman presented with abdominal pain, nausea and vomiting of 24 h duration. She was afebrile and normotensive but had tachycardia. Her upper abdomen was visibly distended and a palpable epigastric mass could be felt. The abdomen was severely tender to palpation and peritoneal signs were elicited. Her past history was significant for pancreas divisum and chronic pancreatitis. Four years prior, she underwent a Whipple procedure as therapy for her pancreatic abnormalities. This required surgical revision 1 year later with a Puestow operation, due to stricture of the previously performed pancreatico-intestinal anastomosis. Ever since, she experienced intermittent abdominal pain, for which she was prescribed strong analgesics, with only partial symptomatic relief. Her white blood cell count was 17 000 cells/mm^3. A computed tomography (CT) scan of the abdomen was obtained, which demonstrated jejunal intussusception, with findings suggesting bowel ischemia (Figure 1).

After fluid resuscitation, the patient was subjected to an exploratory laparotomy. A small amount of ascites was encountered. Two loops of dilated small bowel were found inferior to the transverse mesocolon, each measuring about 10 cm in maximal diameter. These loops were identified to be part of the previously performed Roux-en-Y and Puestow procedures, going towards the stomach, bile duct and the pancreatico-jejunostomy reconstruction. Upon further exploration, an intussusception just distal to the most distal Roux-en-Y connection was found, and about 30 cm of non-perforated necrotic small bowel was identified. The intussusception occurred in an antegrade fashion, which obstructed both Roux-en-Y reconstructions. With care, the intussuscepted intestine was reduced. The necrotic bowel was then resected, and a side-to-side anastomosis between the most distal aspect of the bowel and the Roux-en-Y reconstruction that was directed towards the Puestow procedure was performed.

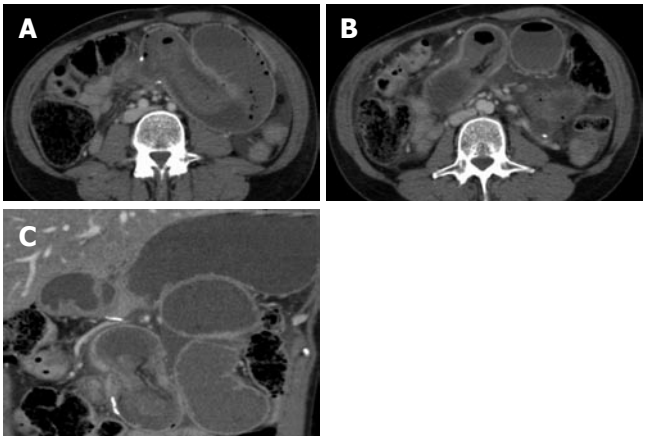

Figure 1 Axial (**A** and **B**) and coronal (**C**) CT images of the abdomen following intravenous contrast administration, which show large dilated loops of small bowel proximal to the intussusception. The intussuscepted bowel entered the more distal jejunum *via* the jejunal anastomotic site, which is evident due to the presence of surgical clips.

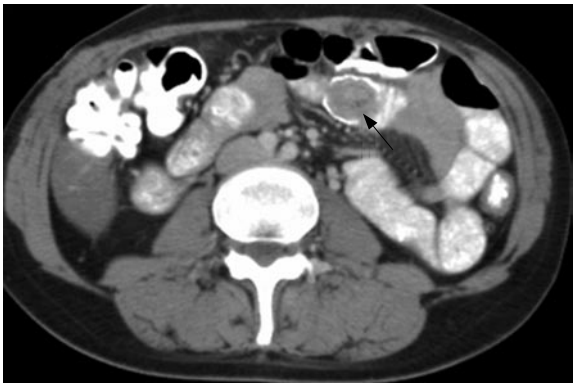

Figure 2 Abdominal CT scan from prior hospital visits, which reveals milder bowel intussusception prior to the patient's last admission.

Due to the massive mesenteric vascular engorgement caused by the intussusception, there was an area of bleeding emanating from a bowel mesentery tear. This was localized and controlled. The abdomen was lavaged and closed. Postoperatively, the patient developed clinical evidence of abdominal compartment syndrome and required emergent re-exploration and blood transfusion. The mesenteric tear was again found to be the source of massive bleeding, and was repaired with additional stitches. Temporary skin closure of the abdomen was performed. Final closure was performed 3 d after the first intervention, and she was discharged without complications 8 d later.

DISCUSSION

Pancreas divisum is an anomaly of the pancreatic ducts, which represents the most common congenital variant of the pancreas. It results from the absence of embryological fusion of the dorsal and ventral pancreatic ducts, each keeping their drainage autonomy[8]. The correlation of this abnormality with pancreatic disease is very controversial[9]. Several techniques have been suggested for therapy, including endoscopic papillotomy, open surgical accessory sphincteroplasty, or a Puestow procedure[10]. As a result of the underlying duct anomalies and significant pancreatic head changes, some have suggested treatment with duodenum-preserving pancreatic head resection (Beger's pancreatectomy)[10]. With good patient selection, the outcome of surgical therapy has been shown to be acceptable.

Our patient underwent a Whipple procedure for chronic pancreatitis, which did not achieve symptomatic relief. This was likely due to stenotic involvement of the entire pancreatic duct, and not only the head portion, as well as due to a stricture at the pancreatico-intestinal anastomosis. This was recorded in the patient's old medical records. In consequence, a Puestow operation was subsequently performed, which resulted in symptomatic improvement but incomplete relief. The latter procedure would have likely been a better first modality of therapy for this patient upon her initial presentation, together with a papillotomy of the minor papilla. However, endoscopic retrograde cholangiopancreatography images were not available to us, and it is therefore impossible to give an accurate opinion about her initial treatment.

The chronic nature of our patient's symptoms made her diagnosis challenging. This was due to the fact that she had recurrent symptoms of abdominal pain, nausea and vomiting after both interventions, and that she required large doses of analgesics and antidepressants due to chronic pain. In fact, previously performed CT scans revealed milder degrees of small bowel intussusception in prior hospital visits (Figure 2), which were thought to represent transient short bowel segment intussusceptions.

It has been suggested that altered intestinal motility may contribute to the development of intussusception[2]. In fact, this complication may be an extreme form of the so-called Roux-en-Y stasis syndrome[3]. It has been shown that the myoelectric activity of the Roux limb is often dysfunctional, split and retrograde, and of high amplitude (> 120 mmHg). Therefore it is possible that the intussusception seen in our patient was the result of severe disruption of the normal pacemaker activity in the intestines[3]. This is even more likely given the fact that we did not identify any intraluminal, extraluminal or intramural lesions. Her current presentation with necrotic bowel did not allow us to perform further imaging studies (i.e., small bowel follow through or gastric emptying studies) to demonstrate altered motility and peristaltic motion, and, rather, mandated emergent exploration.

Cases of small bowel intussusception in adults without a lead point have rarely been reported. They are most often seen after gastric bypass is performed for morbid obesity[4,5], but also have been reported exceptionally after biliary reconstruction for choledochal cysts[6], or associated with *Vibrio* infection in a patient with diabetic ketoacidosis[11]. Intussusception occurring after pancreatic duct reconstruction is extremely rare. It was reported for the first time after a pancreatico-jejunostomy in 2003[7]. The latter case reported retrograde intussusception of the efferent limb into the anastomosis of a revised Roux-en-Y bypass of the pancreas, similar to our case. Our patient represents the second reported case of an

antegrade intussusception that occurred after pancreatic reconstruction. The retrograde case that was reported by Whipple and colleagues[7], occurred after a Roux-en-Y revision for an antegrade intussusception after a Puestow procedure performed for chronic pancreatitis.

A lead point is identified in approximately 80% of intussusception cases[7]. In the current case, we were not able to identify a lead point. Interestingly, neither was this noted in the case reported by Whipple *et al*[7].

Our patient unfortunately developed abdominal compartment syndrome due to massive hemoperitoneum. The massive intestinal dilatation accounted for the friability of the bowel mesentery, which, together with an elevated venous pressure caused by blood flow obstruction in the caval-mesenteric veins, due to the mass effect produced by the bowel obstruction, may explain the large amount of bleeding. Permanent abdominal closure after our second intervention was precluded because of bowel edema and disseminated intravascular coagulation after massive resuscitation, due to the large amount of blood loss.

In conclusion, antegrade intussusception in adults after pancreatic duct reconstruction is extremely rare. Our case represents the second report in the literature of such an occurrence. This patient had previous episodes of abdominal pain, nausea and vomiting, which suggests that altered intestinal motility may have contributed to her current presentation. Bowel intussusception should be always considered in cases of small bowel obstruction in adults after pancreatic reconstruction.

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NEWS

Lian-Sheng Ma, Editor-in-Chief of *WJG*, warmly meets Professor Hugh J Freeman from the University of British Columbia

You-De Chang

You-De Chang, The WJG Press, PO Box 2345, Beijing 100023, China
Correspondence to: You-De Chang, PhD, Science Editor, The WJG Press, Room 1066, Yishou Garden, No. 58, North Langxinzhuang Road, PO Box 2345, Beijing 100023, China. y.d.chang@wjgnet.com
Telephone: +86-10-85381901 Fax: +86-10-85381893
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Abstract

Lian-Sheng Ma, Editor-in-Chief of *World Journal of Gastroenterology* (*WJG*), warmly met Professor Hugh J Freeman from the University of British Columbia at Peninsula Hotel in Beijing on August 28, 2007. Professor Hugh J Freeman gave much helpful advice toward the further development of *WJG*. He will serve as series editor for a new column called OBSERVER which will start in *WJG* in 2008.

Chang YD. Lian-Sheng Ma, Editor-in-Chief of *WJG*, warmly meets Professor Hugh J Freeman from the University of British Columbia. *World J Gastroenterol* 2007; 13(44): 5957

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Professor Lian-Sheng Ma (left), Editor-in-Chief of *WJG*, and Dr. You-De Chang (right) warmly met Professor Hugh J Freeman (middle) at Peninsula Hotel in Beijing. Photograph taken by Mrs. Sally Freeman.

Professor Lian-Sheng Ma, Editor-in-Chief of *World Journal of Gastroenterology* (*WJG*) warmly met Professor Hugh J Freeman, a highly respectable gastroenterologist from the Department of Medicine, University of British Columbia, and his wife Mrs. Sally Freeman during their visit to *WJG* at Peninsula Hotel in Beijing on August 28, 2007. Both sides achieved very fruitful talks and reached a couple of common viewpoints related to future development strategy and management of *WJG*.

Professor Ma gave a detailed introduction to the strategies of both fast peer review and online free access currently taken by *WJG*. "Both fast peer review and online free access are very beneficial and competitive." replied Hugh J Freeman, "It is really just like having a lesson through reading the comments affiliated at the end of an article." He also suggested that *WJG* openly add the names of the peer reviewers at the end of the affiliated comments to make the science communities of authors, reviewers and readers more active and real. "These three aspects have played important roles in ensuring the quality of articles and increasing the public access to *WJG*." he added.

Professor Freeman encouraged with confidence the authors-created, innovation-orientated and readers-benefited publishing system currently conducted by *WJG* with little commercial evolvement. "Over commercial evolvement sometimes misleads the path a journal takes and weakens the decisions a journal makes." Freeman pointed out.

As the second important topic of their talks, Professor Ma invited Professor Freeman to be Associate Editor-in-Chief for a unique column called OBSERVER which will start in *WJG* in 2008. Freeman kindly accepted the invitation. The OBSERVER column will serve as a forum for both gastroenterologists and hepatologists worldwide. Professor Freeman will periodically invite a set of experts from specific research fields to discuss a series of hot topics covering the progress made in both gastroenterology and hepatology, and the challenging questions currently faced by gastroenterologists and hepatologists as well as the possible ideas, ways and techniques to answer these questions. The OBSERVER is an invited editorial for free of publication. For more information, please do not hesitate to contact Professor Freeman at hugfree@shaw.ca and Science Editor Dr. You-De Chang at y.d.chang@wjgnet.com.

On behalf of both Professor Hugh J Freeman and the upcoming OBSERVER column, the *WJG* staff sincerely thank all editorial members, authors and readers from around the world and warmly welcome your coming submissions.

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Emeritus Professor, International Medical Information Center, Shinanomachi Renga Bldg. 35, Shinanomachi, Shinjukuku, Tokyo 160-0016, Japan

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Thomas Bock, PhD, Professor
Department of Molecular Pathology, Institute of Pathology, University Hospital of Tuebingen, D-72076 Tuebingen, Germany

Filip Braet, Associate Professor
Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia

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Parimal Chowdhury, Professor
Department of Physiology and Biophysics, College of Medicine University of Arkansas for Medical Sciences, 4301 W Markham Street Little Rock, Arkansas 72205, United States

Burt Alastair David, Professor
Dean of Clinical Medicine, Faculty of Medical Sciences, Newcastle University, Room 13, Peacock Hall, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, United Kingdom

Francesco Feo, Professor
Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e Oncologia, Università di Sassari, *Via* P, Manzella 4, Sassari 07100, Italy

Robert Flisiak, PhD
Department of Infectious Diseases, Medical University of Bialystok, 15-540 Bialystok, Zurawia str. 14, Poland

Ignacio Gil-Bazo, MD, PhD
Cancer Biology and Genetics Program, Memorial-Sloan Kettering Cancer Center, 1275 York Avenue. Box 241, New York 10021, United States

William Greenhalf, PhD
Division of Surgery and Oncology, University of Liverpool, UCD Building, 5th Floor, Royal Liverpool University Hospital, Daulby Street, Liverpool, L69 3GA, United Kingdom

Yik-Hong Ho, Professor
Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Sherif M Karam, Dr
Department of Anatomy, Faculty of Medicine and Health Sciences, United Arab Emirates University, POBox17666, Al-Ain, United Arab Emirates

Peter Karayiannis
Department of Medicine, Hepatology Section, St Mary's Hospital Campus, South Wharf Road, London W2 1NY, United Kingdom

Peter Laszlo Lakatos, MD, PhD,
Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Thomas Langmann, Associate Professor
University of Regensburg, Institute of Human Genetics, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany

Lucia Malaguarnera, Associate Professor, MD, PhD
Department of Biomedical Sciences, University, Via E. De Amicis, 24 Trecastagni Catania 95039, Italy

Peter J Mannon, MD
Mucosal Immunity Section, Laboratory of Host Defense, National Institute of Allergy, Laboratory of Clinical Investigation, Building 10/CRC, Room 6-3742, 9000 Rockville Pike, Bethesda, Maryland 20892, United States

Basson Marc, MD, PhD, MBA
Chief of Surgery, John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Yoshiharu Motoo, MD, PhD
FACP, FACG,Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University,1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Chris Jacob Johan Mulder, Professor
Department of Gastroenterology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands

Florian Obermeier
Internal Medicine I , University of Regensburg, Franz-Josef-Strauss Allee11, Regensburg 93053, Germany

Jay Pravda, MD
Inflammatory Disease Research Center, Gainesville, Florida 32614-2181, United States

Massimo Raimondo, Dr
Division of Gastroenterology and Hepatology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, United States

Richard A Rippe, Dr
Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Francis Seow-Choen, Professor
Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

Shinji Shimoda, MD, PhD
Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan

Bruno Stieger, Professor
Department of Medicine, Division of Clinical Pharmacology and Toxicology, University Hospital, Zurich 8091, Switzerland

Stefan Wirth, Professor, Dr
Children's Hospital, Heusnerstt. 40, Wuppertal 42349, Germany

Shu Zheng, Professor
Scientific Director of Cancer Institute, Zhejiang University, Secondary Affiliated Hospital, Zhejiang University, 88# Jiefang Road, Hangzhou 310009, Zhejiang Province, China

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symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
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www.cag-acg.org/cddw/cddw2007.htm

Meeting Inflammatory Bowel Diseases 2007
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www.come-innsbruck.at/events/ibd2007/default.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
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Sevilla
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Meeting BSG Annual Meeting
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Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
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Meeting SAGES 2007 Annual Meeting -part of Surgical Spring Week
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Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
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EDITORIAL

Use of second generation contrast-enhanced ultrasound in the assessment of focal liver lesions

Stanislas HX Morin, Adrian KP Lim, Jeremy FL Cobbold, Simon D Taylor-Robinson

Stanislas HX Morin, Adrian KP Lim, Department of Imaging Sciences, Hammersmith Hospital Campus, Faculty of Medicine, Imperial College London, London, United Kingdom
Jeremy FL Cobbold, Simon D Taylor-Robinson, Liver Unit, St Mary's Hospital Campus, Faculty of Medicine, Imperial College London, London, United Kingdom
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Abstract

Ultrasound (US) is often the first imaging modality employed in patients with suspected focal liver lesions. The role of US in the characterisation of focal liver lesions has been transformed with the introduction of specific contrast media and the development of specialized imaging techniques. Ultrasound now can fully characterise the enhancement pattern of hepatic lesions, similar to that achieved with contrast enhanced multiphasic computed tomography (CT) and magnetic resonance imaging (MRI). US contrast agents are safe, well-tolerated and have very few contraindications. Furthermore, real-time evaluation of the vascularity of focal liver lesions has become possible with the use of the newer microbubble contrast agents. This article reviews the enhancement pattern of the most frequent liver lesions seen, using the second generation US contrast media. The common pitfalls for each type of lesion are discussed. The recent developments in US contrast media and specific imaging techniques have been a major advance and this technique, in view of the intrinsic advantages of US, will undoubtedly gain popularity in the years to come.

Key words: Microbubble contrast agents; Ultrasound; Focal liver lesions

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INTRODUCTION

The non-invasive detection and characterisation of focal liver lesions is an important component of cross-sectional imaging studies. Depending on their histological nature, different focal liver lesions vary in their blood supply, with the malignant ones generally having a preferential hepatic arterial supply[1]. The enhancement pattern of a lesion is based on the blood supply and constitutes the mainstay of its characterization with contrast-enhanced computed tomography or magnetic resonance imaging[2].

Ultrasound (US) is often the first imaging investigation for patients with liver disease. The sensitivity and specificity of gray-scale US for the characterization of focal lesions is inferior to that of CT or MRI[3,4]. One of the main reasons for this was the absence, until the 1990s, of US contrast media. The advent of microbubble contrast agents has led to improved characterisation and detection of focal liver lesions since the enhancement characteristics can be visualised in real time over a 5 min period. As a result, recent studies have reported sensitivities and specificities that rival that of CT and MRI[5].

US CONTRAST MEDIA AND SPECIFIC IMAGING TECHNIQUE

Ultrasound contrast agents consist of microbubbles of gas with a protein, lipid or polymer shell. The microbubbles are approximately 1 to 10 μm, which is the size of a red blood cell. These particles are too large to pass through the vascular endothelium and, as such, are considered pure blood pool agents[6]. After several minutes in the circulation, the microbubbles dissolve, the gas is exhaled and the shell is metabolized, mainly in the liver[7]. Furthermore, the microbubbles are well tolerated by patients after intravenous injection and there are very few contraindications to their use.

When subjected to an US wave, the microbubbles respond by changing their size: they expand during the rarefaction phase and contract during the pressure phase. These changes are much greater than the minor changes that occur in the soft tissues. The bubbles, like every oscillating system, have a natural frequency (the resonance frequency) at which their response is maximal.

Fortunately, the bubbles resonate at frequencies used for diagnostic US imaging. This coincidence accounts for their high reflectivity, even when they are present in a small concentration. Furthermore, the expansion of these bubbles during the rarefaction phase exceeds their contraction during the pressure phase. This asymmetric oscillation produces a returning signal (echo) that contains harmonics, i.e. multiples of the driving frequency[8]. The first microbubble-specific imaging technique exploited this property by sending an US pulse into the tissue and selectively detecting echoes at twice that frequency, such that one could theoretically image only the bubbles. However, in practice, soft tissues also produce harmonic echoes. Consequently, the images produced with this technique were of poor quality, in part due to poor tissue suppression.

Subsequently, another mode of imaging with microbubbles was developed using high-power colour Doppler US. When submitted to a high-energy US beam, the microbubbles often break up into much smaller bubbles that dissolve rapidly. As they are disrupted, the bubbles emit a strong, brief echo which is easily detected, a phenomenon known as stimulated acoustic emission (SAE)[9]. The drawback of this technique, however, is that the microbubbles are destroyed by the US beam and therefore real-time imaging is not feasible.

At the present time, the most common imaging technique is based on the principle of phase-inversion, in which two US pulses, 180° out of phase, are sent sequentially. The returning echoes are added up by the US machine[10]. The linear echoes returned by the tissues nullify each other, while the non-linear echoes returned by the microbubbles produce a detectable signal. There are two main advantages of this technique. First, excellent tissue suppression is obtained. Second, a detectable signal is obtained even when a very low-power US beam is employed, consequently, the bubbles are not destroyed. The first generation contrast media, such as Levovist® (Schering, AG, Berlin, Germany), produced a very weak signal when submitted to low mechanical index US beam owing to its fragility and lent itself to use with SAE[11]. Since then, contrast media, such as SonoVue® (Bracco, Milan, Italy), Definity® (Bristol-Myers-Squibb, Billerica, Mass, USA) and Optison® (Nycomed/Amersham, Little Chalfont, UK), which have a strong, non-linear, harmonic response, even when insonated with low acoustic power, were able to provide real-time imaging using low mechanical index (MI) modes[12].

In the following sections we review the common enhancement patterns of the most frequent focal liver lesions seen with the second generation contrast agents.

CONTRAST-ENHANCED US PATTERNS OF FOCAL LIVER LESIONS

The characterization of a hepatic lesion with microbubbles requires careful examination through all phases of contrast enhancement, i.e. arterial (10-20 to 25-35 s after injection), portal (30-45 to 120 s) and late parenchymal (> 120 s) phases[13]. Simply put, the late phase is useful to determine the benign or malignant nature of a lesion while the arterial phase helps in predicting its histology[14-19]. Between 86% to 93% of benign lesions retain the contrast in the late phase, while 78% to 98% of the malignant ones demonstrate wash out of the contrast[14,16,18]. The persistence of the second generation contrast agent in a healthy liver is thought to be the result of a very slow flow through the sinusoid[20]. Consequently, lesions devoid of normal sinusoids do not retain the contrast.

HAEMANGIOMAS

Haemangiomas are the most common solid benign lesion of the liver, with a prevalence ranging from 1% to 20% in the general population[21]. These lesions are more common in females and are frequently located peripherally or adjacent to a large hepatic vein branch. The most common sonographic appearance of haemangiomas is a homogeneously hyperechoic focal lesion, less than 3 cm in size[22]. These characteristic features, when present in a patient at a low risk for malignancy, are usually sufficient to allow a confident diagnosis. In a significant number of patients however, further imaging is required.

The characteristic early arterial nodular enhancement with delayed centripetal fill-in described on CT or MRI[23-25] is the most common appearance of haemangiomas during the arterial phase of contrast-enhanced US, seen in 52% to 88% of cases (Figure 1)[15,16,18,19]. Sustained enhancement has been reported in 83% to 100% during the late phase[16-19]. The real time nature of contrast-enhanced US is particularly useful in diagnosing small, rapidly perfusing (flash-filling) haemangiomas, where the typical enhancement pattern can be appreciated[6]. Complete enhancement does not always occur, especially in lesions larger than 3 cm, which often undergo central thrombosis or fibrosis[6,26,27].

FOCAL NODULAR HYPERPLASIA

Focal nodular hyperplasia (FNH) is the second most common solid benign hepatic lesion, with a prevalence between 0.9% and 3%[28-30]. This lesion occurs in all age groups and both sexes, but is found predominantly in women (80%-95% of the cases) during the 3rd to 5th decade of life. Oral contraceptive use has been incriminated, but a definite relationship has not been established[31,32]. FNH is not a dysplastic or neoplastic tumour, but is a hyperplastic lesion, probably occuring in response to a pre-existing arterial malformation[33]. FNH does not have a malignant potential, nor is it likely to bleed or rupture[34]. Consequently, differentiation from other lesions, particularly hepatocellular adenoma and carcinoma (especially the fibrolamellar form), is essential since FNH is managed conservatively, whereas the other lesions require surgery. FNH is often discovered incidentally.

The most common sonographic appearance of FNH is that of a homogeneous, near isoechoic lesion; some lesions are detected only because of their mass effect on adjacent blood vessels[32]. A central hypoechoic scar is detected in 20% to 45% of the cases[22,35,36]. In larger lesions, colour Doppler may show a central feeding artery with a spoke-

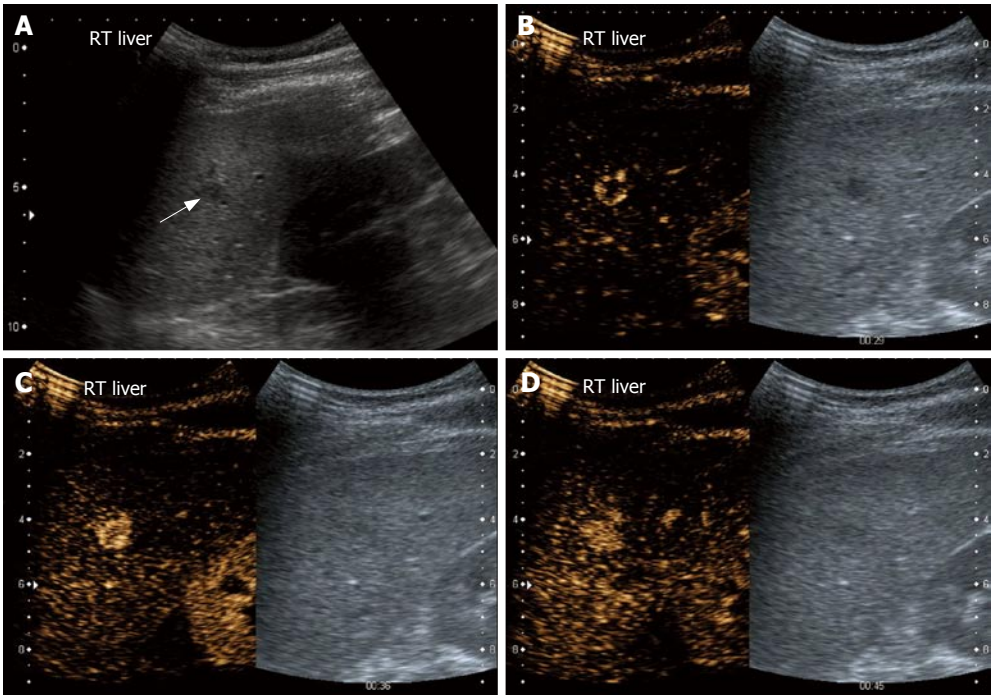

Figure 1 Haemangioma. Gray-scale US image (**A**) and split-screen display images of contrast-enhanced US scan using a low MI technique (**B-D**). The left panel shows the contrast sensitive image while the corresponding gray-scale image is on the right. On gray-scale US, the liver is hyperechoic, consistent with fatty infiltration and an ill-defined hypoechoic lesion is seen (arrow, **A**). On contrast enhanced US, the lesion demonstrates peripheral nodular enhancement during the arterial phase (**B**). At 36 s, the lesion has almost completely filled in (**C**). At 45 s, the lesion is completely enhanced (**D**) and sustained enhancement was observed in the late phase scan (not shown).

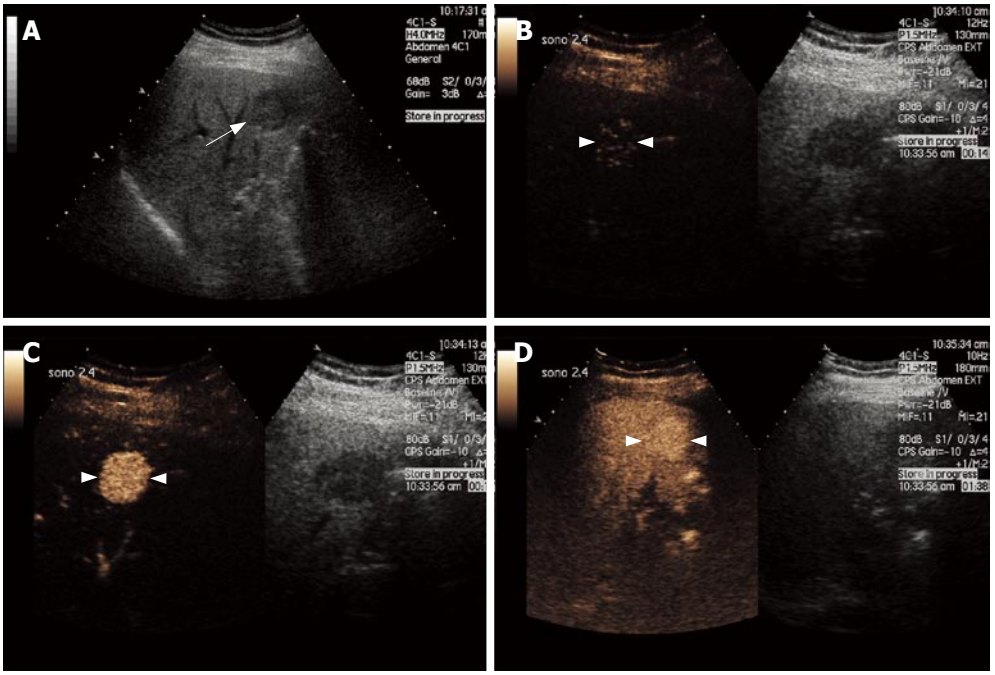

Figure 2 Focal Nodular Hyperplasia. Gray-scale US image (**A**) and split-screen display images of a contrast-enhanced US scan using a low MI technique (**B-D**). The gray-scale US image shows a focal hypoechoic lesion (arrow, **A**) in a diffusely hyperechoic liver in keeping with fatty infiltration. After contrast injection, the lesion enhances avidly in the arterial phase with filling seen from a central feeding vessel, demonstrating the classical spoke-wheel appearance (arrowheads, **B** and **C**). The lesion remains slightly hyperechoic during the portal and late phases (arrowheads, **D**).

wheel pattern of vessels radiating to the periphery.

FNH is a hypervascular tumour and consequently manifests as a strongly and homogeneously enhancing lesion during the arterial phase of contrast enhanced US in nearly 100% of the cases (Figure 2). The central spoke-wheel type of contrast enhancement can be demonstrated in 45% to 89% of FNH. These lesions become isoechoic or slightly hyperechoic, compared with the surrounding liver parenchyma during the portal and late phases of enhancement in 87% to 100% of the time[15-19,37]. The central scar is seen in 23% to 31% of cases[15,17]. However, in contradistinction to the pattern seen on CT or MRI, the central scar stands out as a defect instead of the late enhancement seen with the other imaging modalities. This finding can be explained by the fact that microbubbles are purely intravascular agents and therefore do not diffuse into the interstitium, unlike iodine and gadolinium-based contrast agents used with CT and MRI[6].

In patients with chronic liver disease, caution should be used since well-differentiated hepatocellular carcinoma (HCC) may mimic the enhancement pattern of FNH (see below). In these patients, all hypervascular lesions should be regarded with suspicion.

HEPATOCELLULAR ADENOMA

Hepatocellular adenoma (HA) is a rare, benign neoplasm of hepatocellular origin. Approximately 90% of HAs occur in young women[38]. Up to 90% of females with HA have reported the use of oral contraceptives[39]. HA is also

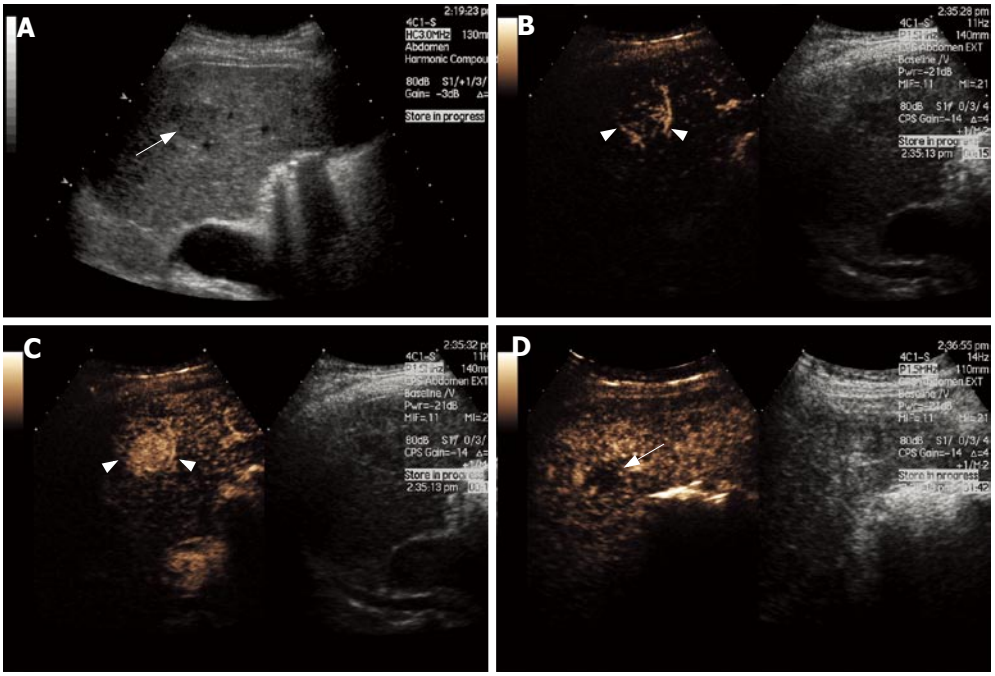

Figure 3 Hepatocellular Carcinoma. Gray-scale US image (**A**) and split-screen display images of a contrast-enhanced US scan using a low MI technique (**B-D**). The gray-scale US image shows a slightly hypoechoic lesion in segment 2 of the liver (arrow, **A**). After contrast injection, the lesion shows marked hypervascularity in the arterial phase with a basket-pattern peripheral network of vessels (arrowheads, **B** and **C**). The lesion becomes isoechoic and finally washes out to leave a defect in the late phase (arrow, **D**).

associated with the use of anabolic steroids in men and in some storage diseases[40]. In contrast to FNH, HA is a true neoplasm.

Management of HA is by surgical resection, in contrast to FNH, because of the risk of malignant degeneration and haemorrhage[41]. Patients with HA may present with pain secondary to its mass effect (40%) or intratumoral/intraperitoneal haemorrhage (40%). Alternatively, the tumour may be discovered incidentally (20%)[22].

The most common sonographic appearance of HA is a well-defined, large, solitary, hyperechoic mass owing to its high lipid content. Since the lesions have a propensity to bleed, HAs are usually heterogeneous in appearance. Colour Doppler findings are non-specific. The typical spoke-wheel pattern of FNH is absent.

The contrast-enhanced US characteristics of HA are relatively non-specific, but these lesions usually enhance during the arterial phase. Smaller lesions are likely to show homogeneous enhancement whereas the larger ones will be heterogeneous owing to previous intratumoral haemorrhage or necrosis. In one study with Sonovue, HAs were iso- or more often hypoechoic in comparison with the surrounding liver parenchyma in the portal venous and late phases of enhancement[37]. Unfortunately, this pattern of enhancement is not unique to HA, as it is also a common appearance of HCC on contrast-enhanced US (see below). In some cases, even the histopathological differentiation of HA from well-differentiated HCC is difficult[42]. The differential diagnosis should include hypervascular metastases which can exhibit similar enhancement characteristics.

HEPATOCELLULAR CARCINOMA

HCC is the most common primary malignancy of the liver. It usually occurs in patients with chronic liver disease, particularly in those with chronic hepatitis B and C infection where the risk is approximately 100 times that of patients with cirrhosis of other aetiologies. Men are affected three times more frequently than women[43]. Early detection is crucial for curative treatment, since patients with small HCC (< 2 cm) who are treated with liver transplantation have a survival rate of about 80%[44], whereas the 5-year survival of untreated HCC is less than 5%.

The gray scale US appearance of HCC is variable and non-specific. Hyperechoic foci related to the presence of fibrosis, haemorrhage and necrosis are found in approximately 50% of large HCCs[22]. On colour Doppler, approximately 75% of HCCs have a fine peripheral network of vessels, surrounding and penetrating the lesion (the so-called basket pattern)[45]. Non-invasive imaging diagnosis of HCC is often based on CT or MRI detection of a hypervascular mass in a patient with chronic liver disease because of the lack of specificity of conventional US.

With the use of contrast-enhanced US, more than 90% of HCCs behave like other hypervascular lesions and enhance avidly during the arterial phase (Figure 3)[15,17-19]. The basket pattern is reportedly seen in about one-third of the cases[19]. Also, like other malignant lesions, the majority (83% to 97%) of HCCs washout the contrast and appear as a defect during the late phase. However, caution should be used since well-differentiated HCCs may not show this washout very reliably. Moreover, it has been observed that the more differentiated a lesion, the more slowly it is likely to washout[46]. Consequently, in a patient with known chronic liver disease, a hypervascular lesion in the arterial phase should not be considered as benign on the sole finding of persistent enhancement during the portal and late phases.

METASTASES

The liver is a frequent site of metastases of extrahepatic tumours, and metastatic disease is one of the most common indications for imaging the liver. The gray-scale sonographic appearances of metastases are varied

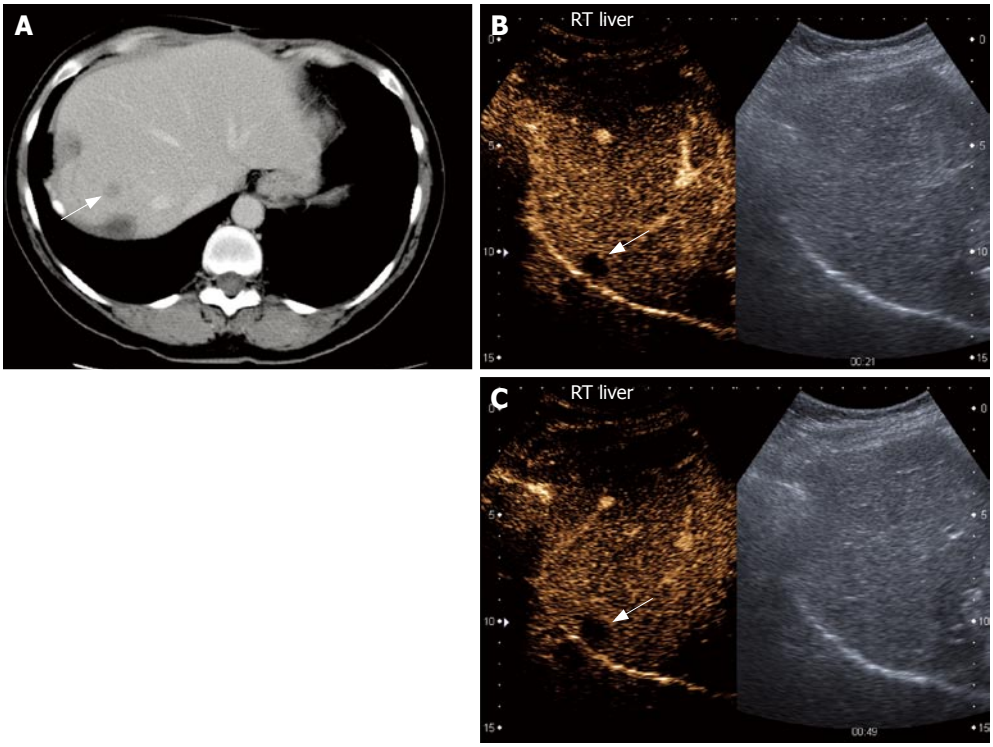

Figure 4 Liver metastasis in a patient with known metastatic colon carcinoma. The patient previously had liver resection and radiofrequency ablation. Contrast-enhanced CT scan image (**A**) and split-screen display images of a contrast-enhanced US scan using a low MI technique (**B** and **C**). On CT, a 1cm lesion is seen in segment 8 (arrow, **A**). The lesion was not visualized on gray-scale US. After injection of microbubbles, a 1 cm hypoechoic rounded lesion is seen as a defect in all the phases of enhancement (arrow, **B** and **C**). These findings are suspicious for a metastatic deposit.

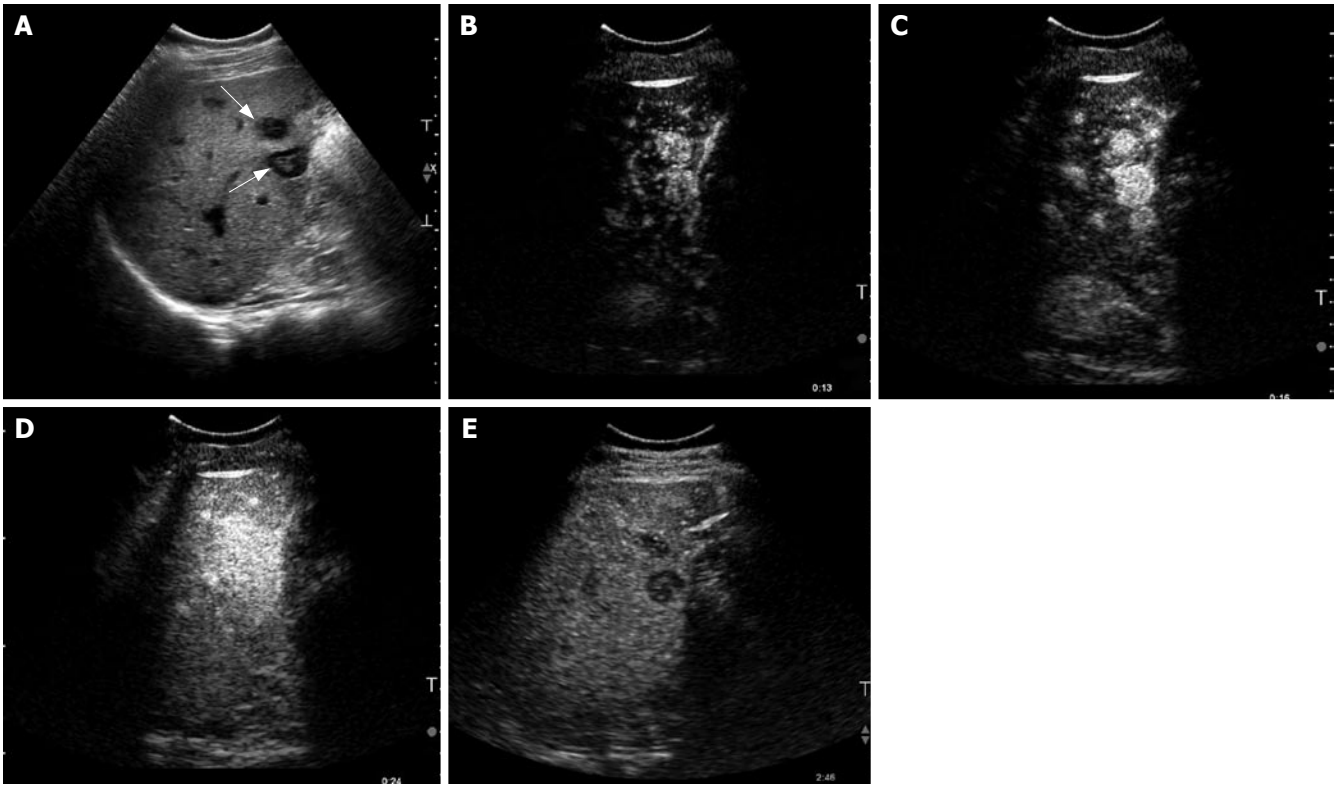

Figure 5 Hypervascular metastases in a patient with a known carcinoid tumour. Gray-scale US image (**A**) and contrast-enhanced US scan images (**B**-**E**) using a microbubble sensitive technique. On the gray-scale image, two hypoechoic target-like lesions are seen (arrows, **A**). After microbubble enhancement, an avid uptake of the contrast medium was seen in the early phase (**B**-**D**). The contrast washed out in the later phases leaving the metastases as defects (**E**).

depending on several factors such as the histology of the primary tumour and the treatment received by the patient. In a patient with a known malignancy with interval development of hepatic masses, the diagnosis is straightforward and characterisation is not an issue. However, when there is no history of malignancy or no previous imaging for comparison, characterisation becomes essential.

The accuracy of US for the assessment of liver metastases is lower than that of CT or MRI[47]. However, the use of first generation US contrast media, improved significantly the sensitivity for the detection of liver

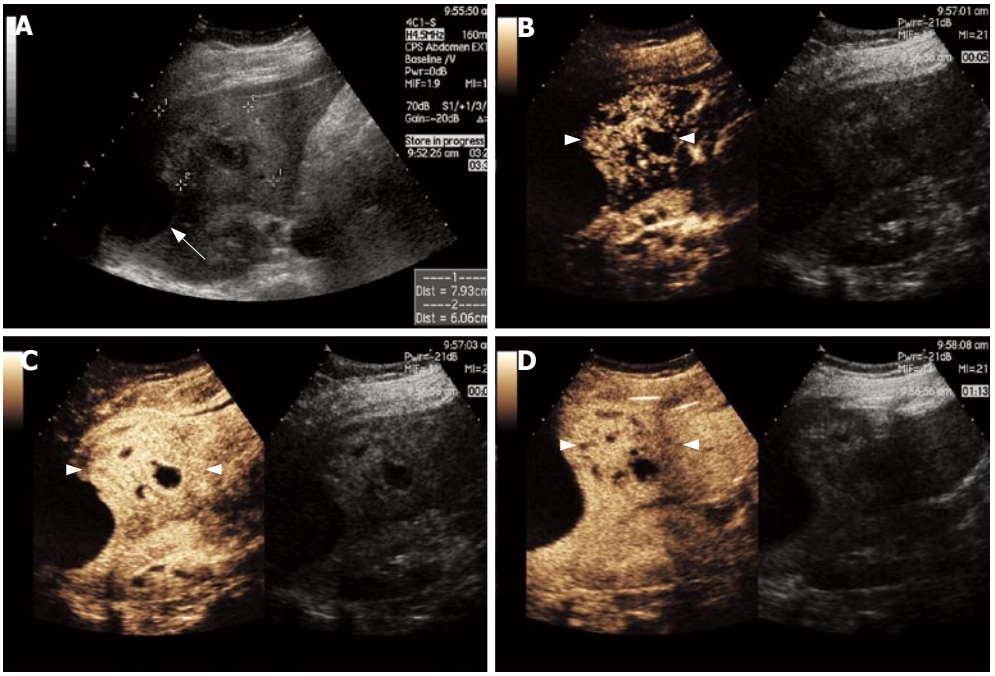

Figure 6 Hepatic abscess. Gray-scale US image (**A**) and split-screen display images of a contrast-enhanced US scan using a low MI technique (**B**-**D**).The gray-scale image shows an ill-defined heterogeneous mass in the left lobe of the liver (between callipers, **A**). A subcapsular anechoic fluid collection is also seen (arrow, **A**). After microbubble injection, regional hypervascularity during the arterial phase is shown (arrowheads, **B**-**D**). The abscess appears as a cluster of non-enhancing collections separated from each other by enhancing septations (**B** and **C**). In the late phase scan (**D**), there is no enhancement of the fluid collections and no wash out of the enhancing portions.

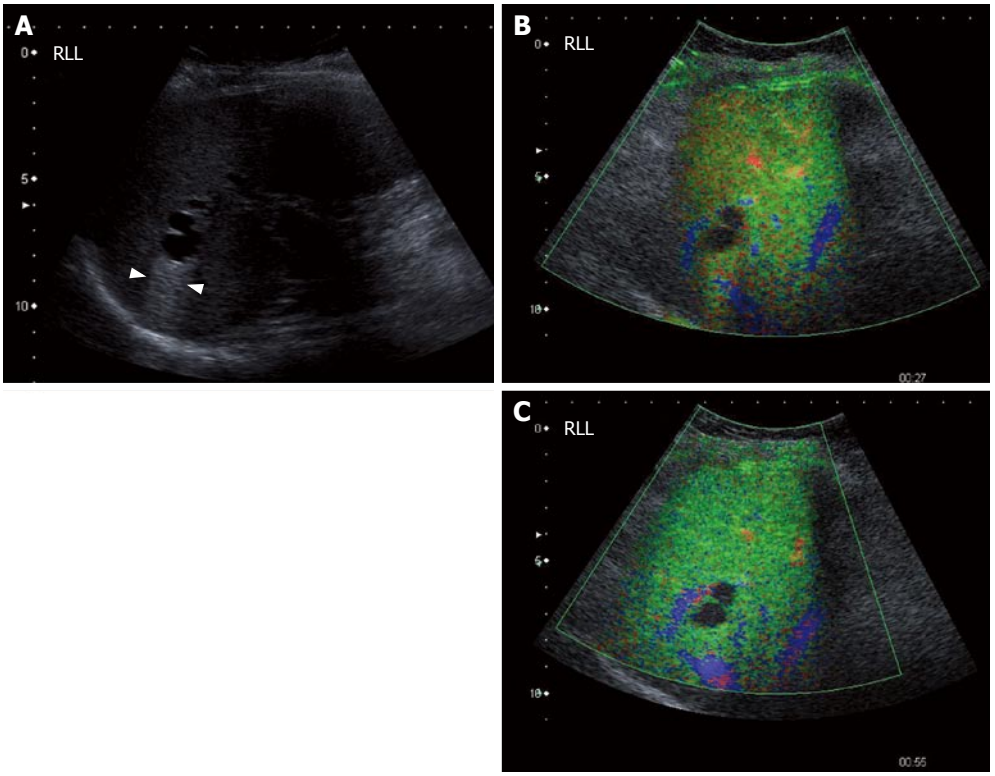

Figure 7 Potential pitfall: simple cysts. Gray-scale US image (**A**) and contrast-enhanced US scan images using a low MI technique (**B** and **C**). The gray-scale image (**A**) shows a typical simple hepatic cyst which is completely anechoic, has a thin wall and posterior acoustic enhancement (between arrowheads, **A**). After microbubble injection, no enhancement of the cyst is seen throughout all phases of enhancement (**B** and **C**). The diagnosis is straightforward if the lesion was recognised prior to contrast injection. If not, it may be misinterpreted as an enhancement defect and be categorized as a malignant lesion.

metastases[48]. It has been proposed by the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) that any US staging study of the liver should be contrast enhanced, although the cost and feasibility of such a recommendation remains a big hurdle[13].

On contrast-enhanced US, metastases show different patterns of enhancement during the arterial phase, depending upon the vascularity pattern of the primary tumour. Regardless of their behaviour during the early phase, metastasis consistently show rapid and complete contrast washout and appear as enhancement defects on late phase scans (Figure 4)[15-17,19]. Recent publications have shown that, with the second generation US contrast media, the vast majority (> 85%) of metastases show some arterial enhancement, often more pronounced in the periphery[15,16,19,49]. This phase of hypervascularity is often not recognized on multiphasic CT or MRI because it is very brief and the lesion starts to washout within 20 s of the injection in most cases, before the arterial phase of CT and MRI, which is around 40 s from the beginning of the injection. The arterial enhancement may be rim-like,

diffuse or mosaic-like. Rim-like enhancement and early and complete washout of the lesion are typical of metastasis, while complete enhancement with a later washout is most suggestive of a HCC. However, when a hypervascular metastasis shows complete enhancement (Figure 5), differentiation from HCC is difficult and correlation with the clinical history and alpha-fetoprotein is often helpful.

OTHER MALIGNANT LESIONS

Other primary malignant lesions of the liver, such as intra-hepatic cholangiocarcinoma and lymphoma, show the typical behaviour of malignant lesions and washout contrast rapidly and appear as defects on the portal and late phases.

Cholangiocarcinoma frequently enhances during the arterial phase[40,49]. The rapid washout of this lesion observed on contrast-enhanced US is discordant with the typical behaviour of the lesion seen on CT and MRI (hypoenhancement with mild peripheral centripetal progression of enhancement over time)[50]. Again, this may be explained by the fact that microbubbles are purely intravascular agents. Some gray-scale features may help in differentiating cholangiocarcinoma from other abnormalities such as biliary duct dilatation.

ABSCESS

Liver abscesses result from bacterial, amoebic or fungal infections. Pyogenic abscess are by far the most frequent (88%)[22]. The gray-scale US findings of pyogenic abscesses vary with the stage of the disease. During early disease, the shape of the lesion is usually irregular and the echogenicity is variable. As the abscess matures the lesion becomes more rounded and hypoechoic, with debris in the middle and thick walls on the outside. Since an abscess is a fluid-filled lesion, there is usually associated posterior enhancement. Internal septations are seen commonly. Bright punctate echoes with "dirty" shadowing are present if there is gas within the cavity.

On contrast-enhanced US, pyogenic hepatic abscesses show areas of increased enhancement relative to the surrounding parenchyma[51]. Mature lesions with fluid show an enhancing rim. The internal septations also show enhancement giving the lesion a honeycomb appearance. Early (solid-appearing) lesions usually enhance diffusely, but heterogeneously. The enhancement appears early and usually persists during the portal and late phases (Figure 6), with no contrast enhancement seen in the liquefied portions. In the arterial phase, a transient peri-lesional enhancement has been reported. In a minority of cases, this is followed by portal venous phase hypovascularity[51]. The main differential diagnosis for a hepatic abscess is a necrotic metastasis. The latter would appear as a punched-out enhancement defects in the late phase, while the former appears as an ill-defined area of decreased enhancement, although, as stated above, this is not a common finding.

LIVER CYSTS

Liver cysts are common incidental findings on liver US. The diagnosis is straightforward when their pathognomonic features (anechoic, thin-walled and posterior enhancement) are present on gray-scale US.

Liver cysts are mentioned in this review because, on contrast-enhanced US, these lesions represent a potential pitfall if they have not first been recognized on gray scale US, since their gray-scale appearances are essential for characterisation. Liver cysts present as enhancement defects on all phases of contrast-enhanced US scan (Figure 7) and can be erroneously mistaken as malignant lesions.

CONCLUSION

The introduction of second generation microbubble US contrast media has allowed real-time imaging of a liver lesion in every phase of enhancement. The ability to observe the complete pattern of enhancement of a lesion has improved significantly the specificity of US for focal liver lesions, and rivals that of CT and MRI, thus reducing the need for further investigations. As a screening tool, US is ideal owing to its relative accessibility and portability. Microbubble agents have extended the utility of US further and are applicable to most imaging departments worldwide.

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EDITORIAL

Endoscopic pancreatic duct stent placement for inflammatory pancreatic diseases

Pier Alberto Testoni

Pier Alberto Testoni, MD, Division of Gastroenterology, Vita-Salute San Raffaele University, Scientific Institute San Raffaele, Milan 20132, Italy
Correspondence to: Pier Alberto Testoni, MD, Head, Division of Gastroenterology & Gastrointestinal Endoscopy, Vita-Salute San Raffaele Unversity - Scientific Institute San Raffaele, Via Olgettina 60, Milan 20132, Italy. testoni.pieralberto@hsr.it
Telephone: +39-2-26432756 Fax: +39-2-26433491
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Abstract

The role of endoscopic therapy in the management of pancreatic diseases is continuously evolving; at present most pathological conditions of the pancreas are successfully treated by endoscopic retrograde cholangio-pancreatography (ERCP) or endoscopic ultrasound (EUS), or both. Endoscopic placement of stents has played and still plays a major role in the treatment of chronic pancreatitis, pseudocysts, pancreas divisum, main pancreatic duct injuries, pancreatic fistulae, complications of acute pancreatitis, recurrent idiopathic pancreatitis, and in the prevention of post-ERCP pancreatitis. These stents are currently routinely placed to reduce intraductal hypertension, bypass obstructing stones, restore lumen patency in cases with dominant, symptomatic strictures, seal main pancreatic duct disruption, drain pseudocysts or fluid collections, treat symptomatic major or minor papilla sphincter stenosis, and prevent procedure-induced acute pancreatitis. The present review aims at updating and discussing techniques, indications, and results of endoscopic pancreatic duct stent placement in acute and chronic inflammatory diseases of the pancreas.

Key words: Chronic pancreatitis; Pancreas divisum; Pancreatic pseudocyst; Pancreatic fistulas; Idiopathic recurrent pancreatitis; Main pancreatic duct stenting; Pancreatic dorsal duct stenting

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INTRODUCTION

The role of endoscopic therapy in the management of pancreatic diseases is continuously evolving; at present most pathological conditions of the pancreas are successfully treated by endoscopic retrograde cholangio-pancreatography (ERCP) or endoscopic ultrasound (EUS), or both. After the initial courageous experimental therapy tested in a few centers, pancreatic endotherapy has become an evidence-based method for carefully selected patients; because of the high level of technical skill required and the small numbers of patients who need this approach, pancreatic endotherapy should ideally only be carried out in selected centers where a multidisciplinary team is available.

Endoscopic placement of stents has played and still plays a major role in the treatment of chronic pancreatitis, pseudocysts, pancreas divisum, main pancreatic duct injuries, pancreatic fistulae, complications of acute pancreatitis, recurrent idiopathic pancreatitis, and in the prevention of post-ERCP pancreatitis. These stents are currently routinely placed to reduce intraductal hypertension, bypass obstructing stones, restore lumen patency in cases with dominant, symptomatic strictures, seal main pancreatic duct disruption, drain pseudocysts or fluid collections, treat symptomatic major or minor papilla sphincter stenosis, and prevent procedure-induced acute pancreatitis.

On the assumption that intraductal hypertension caused by obstructive lesions of the main pancreatic duct (MPD) is one cause of the pain often present in either chronic or acute pancreatic diseases, stent insertion beyond the obstruction to decompress the hypertension has a pivotal role in their therapeutic management.

The present review aims at updating and discussing the role of endoscopic pancreatic duct stent placement in acute and chronic inflammatory diseases of the pancreas.

TECHNIQUE OF PANCREATIC STENT PLACEMENT

The technique employed for placing pancreatic stents is similar to that used to place stents in the biliary tract. Once the main or accessory pancreatic duct has been deeply cannulated, a hydrophilic 0.035" (for 5F, 7F, 10F stents) or 0.018" (for 3F stents or when the minor papilla is cannulated) guidewire is introduced into the duct and maneuvred if possible beyond the stricture or leakage. The stent is then introduced over the guidewire (Figure 1). Stents can be placed with or without pancreatic sphincterotomy; pancreatic sphincter can be ablated by using the standard sphincterome in a single step procedure or after biliary sphincterotomy. The multiple step procedure is more time consuming but permits to better control the section of the

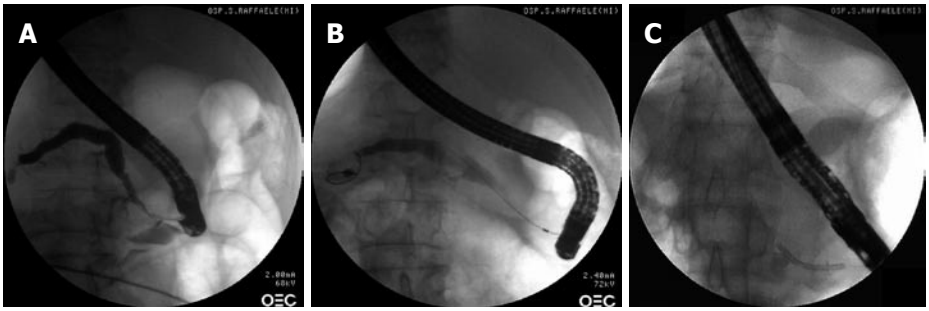

Figure 1 Chronic obstructive pancreatitis involving the head of the gland. **A**: Pancreatography showing a distal stricture of the main pancreatic duct; **B**: Insertion of the guidewire into the main pancreatic duct; **C**: Placement of a 10F plastic stent over the guidewire.

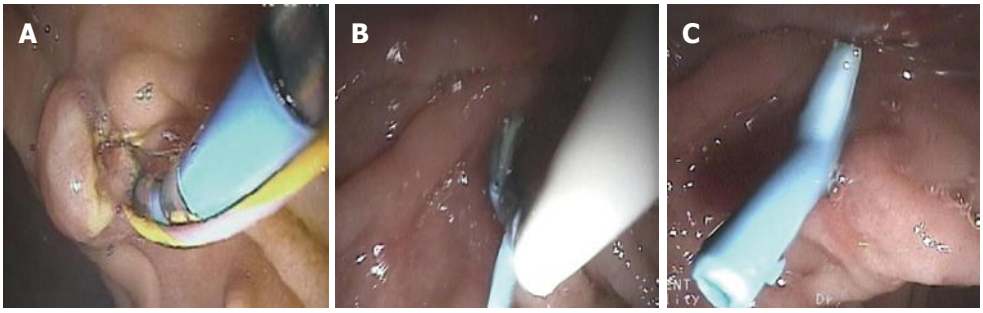

Figure 2 Placement of a pancreatic stent after pancreatic sphincterotomy. **A**: Once biliary sphincterotomy has been performed leaving a guidewire into the common bile duct, pancreatic sphincter ablation is done with the standard sphincterotome; **B**: A plastic stent is then pushed over the guidewire into the main pancreatic duct; **C**: Pancreatic stent in place.

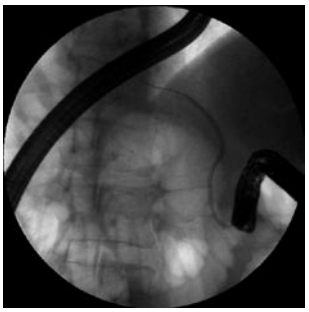

Figure 3 Chronic obstructive pancreatitis involving the head of the gland: insertion of an s-shaped stent into the main pancreatic duct.

sphincter so it is generally my preferred approach (Figure 2).

Pancreatic stents are generally made of polyethylene and are similar to biliary stents except for side holes along their length to allow flow from side branches. To prevent migration into the pancreatic duct, small-diameter stents have a J or "pig-tail" shape. For transpapillary stenting of a pseudocyst, a double pig-tail stent should be used to prevent displacement outside the cyst cavity. Recently, an S-shaped stent with many side holes has been proposed for MPD stenting in chronic pancreatitis[1]; this stent is made of ethylene vinyl acetate, which is more flexible than polyethylene. The S-shape enables the stent to adapt better to the course of the MPD and reportedly achieves a better outcome in patients with chronic pancreatitis and upstream duct dilatation than in patients treated with the standard straight polyethylene stents (Figure 3).

The diameter of the stent should not exceed the size of a normal downstream duct, so 5F and 7F stents should be used in cases with non-dilated ducts, while 10F and sometimes 11.5F can be used when the ducts are dilated, as in advanced chronic pancreatitis. Sometimes in advanced chronic disease the stricture is too tight to place a stent across it; in these cases the stricture must be dilated with a balloon or bouginage to permit insertion. In some cases the Soehendra stent retriever (5F or 8F) can be used to dilate the stricture and allow insertion.

How long stents are best left in place is not yet known. Pancreatic stents have been left in place for six months and long-term therapy requires multiple stent exchange. However, the duration of a single stent placement depends on the stent diameter: the larger the diameter, the longer the stent can be left in place.

CHRONIC PANCREATITIS

In chronic pancreatitis the MPD may be partially occluded by strictures or stones; the rise of intraductal pressure in the ductal segment above the obstruction causes dilation and obstructive pain. Pancreatic intraductal hypertension occurs regardless of the etiology and whether or not the MPD is dilated; ductal and interstitial hypertension, together with reduced acinar blood flow, may further contribute to the formation of fibrosis and progression toward more severe damage[2]. Removing the barriers to outflow of pancreatic juice may relieve chronic pain or exacerbation of chronic pancreatitis. Obstruction-related reduced outflow of pancreatic juice into the duodenum may also cause mal-digestion of nutrients even in cases with still conserved pancreatic enzyme secretion, or worsening of mal-digestion already present in advanced cases.

Although pancreatic ductal strictures can be treated by catheter or balloon dilation alone, a stent usually has to be inserted because stricture relapse is commonplace. Insertion of a stent beyond the ductal blockage achieves lasting relief of the intraductal hypertension and subsequent pain and possible mal-digestion, also restoring the lumen patency, by dilating the stricture. If a 10F stent or larger is used, the patient generally requires sphincterotomy of both the pancreatic and biliary segments of the sphincter, followed by stricture dilation (Figure 4).

The presence of both obstruction and ductal dilation is vital for predicting which patients are most likely to benefit

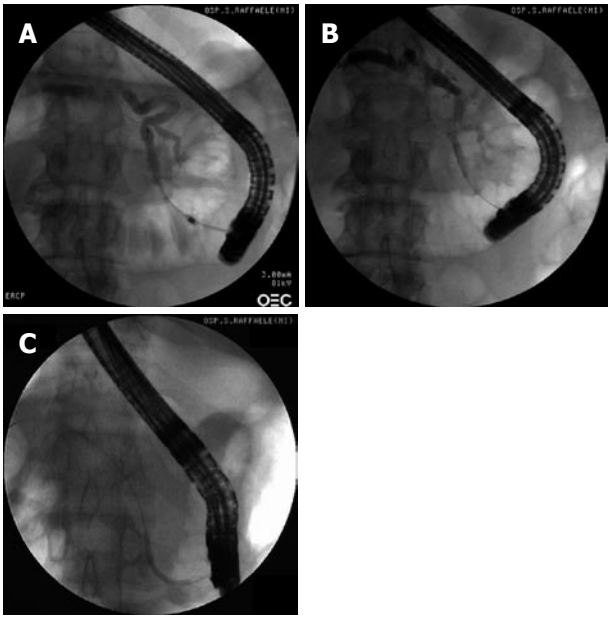

Figure 4 Symptomatic, chronic obstructive pancreatitis at early stage with moderate dilation of the main pancreatic duct. **A**: Mechanical dilation of the pre-papillary stricture by a 10F dilator; **B**: Insertion of the guidewire; **C**: Insertion of a plastic 10F stent throughout the dilated stricture over the guidewire.

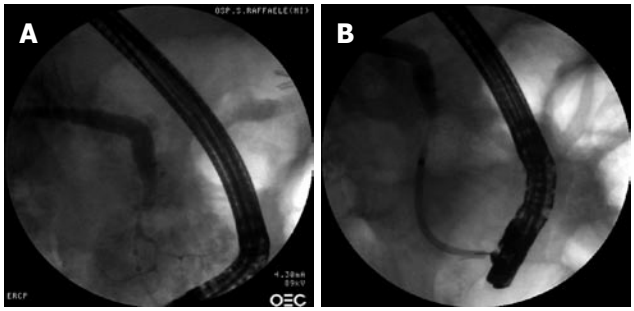

Figure 5 Chronic obstructive pancreatitis. **A**: Severe stricture of the main pancreatic duct with marked upstream dilation; **B**: Placement of a 10F plastic stent after mechanical dilation of the stricture.

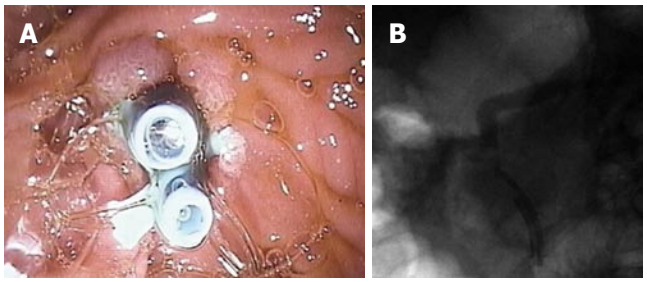

Figure 6 Refractory dominant stricture of the main pancreatic duct in chronic pancreatitis patient already treated by dilation and temporary stenting. Placement of two plastic stents. Endoscopic (**A**) and radiological (**B**) features.

from stricture therapy: the best candidates for stenting are those with a distal stricture and upstream dilation (Figure 5).

Most investigators and recent guidelines from the American Society for Gastrointestinal Endoscopy consider endoscopic management to be the preferred interventional approach for chronic pancreatitis in patients selected on the basis of anatomical changes caused by the disease; endoscopic treatment is generally safe, minimally invasive, can be repeated, and does not interfere with eventual surgery[3,4]. Other investigators, however, found surgery superior to endotherapy for long-term pain reduction. Dite *et al*[5], in a prospective randomized trial comparing endoscopic and surgical therapy for chronic, painful, obstructive pancreatitis, reported complete resolution of pain at the five-year follow-up in 37% of patients after surgery and in 14% of those after endotherapy; short-term results were similar in the two groups. Similar data have been recently published by Cahen *et al*[6].

The technical success of endoscopic stricture manipulation can range from 80% to 100% of patients with or without prior pancreatic sphincterotomy. In chronic pancreatitis patients with dominant stricture, pain relief was obtained in 52%-95% of cases over a follow-up ranging from 8 to 72 mo[1,7-19]. Stenting was also associated with weight gain and fewer hospital visits. Good clinical outcomes were related to cessation of alcohol consumption and/or smoking[15]. Early complications were reported in about 17% of cases and were related mainly to pancreatic and/or biliary sphincterotomy, stent clogging (juice infection) and inward migration.

It is not clear how long stents should best be left in place. Although the plastic 10F stents are thought to remain clinically patent for a year on average, generally they are removed and replaced every 6-9 mo. In fact, stent dysfunction leading to pancreatitis, recurrent pain or infection can occur before the scheduled exchange time in about half the cases, so repeated stent exchange is required in the long term. This may make it difficult to ensure compliance with long-term stenting treatment.

Despite encouraging medium- and long-term results, duct stricture may persist or recur after removal of a stent so definitive removal seems impracticable in a subset of patients, because of the recurrence of pain. In an intention-to-treat analysis, a German multicenter study on long-term outcomes in 1000 patients with chronic pancreatitis after pancreatic stenting reported unsatisfactory results in 35%; 16% of these patients continued with endotherapy and 24% opted for surgery[11].

A multiple stenting approach was proposed by Costamagna *et al*[20] in a subset of patients with refractory dominant MPD strictures: they reported lasting stricture dilation in 84% of their patients at 38-mo follow-up. Although placing a mean of three stents within pancreatic strictures may be difficult, this approach appears feasible and safe and could in fact dramatically reduce the need for surgery in the majority of patients with chronic obstructive pancreatitis (Figure 6).

Self-expandible metal stents have been proposed for patients with relapsing dominant strictures to achieve long-term stent patency and avoid the need for stent exchange[21]. The success rate of stent placement was 100% and patients enjoyed immediate relief of symptoms and reduction of duct diameter; however, during follow-up these patients have had high occlusion rates of the stent from mucosal hyperplasia, and it becomes impossible to remove the stent, so this approach has in fact been abandoned.

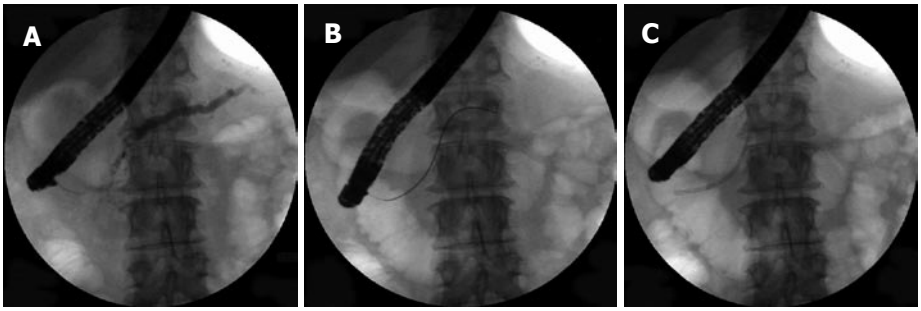

Figure 7 Pancreas divisum with chronic obstructive pancreatitis at early stage. **A**: Extensive dorsal duct stricture with moderate upstream dilation of the pancreatic duct; **B**: Guidewire insertion into the dorsal duct throughout the minor papilla, without sphincterotomy; **C**: 7F plastic stent in place. Long-term stenting rather than minor papilla sphincterotomy appears an appropriate approach in this case with extensive dorsal duct stricture.

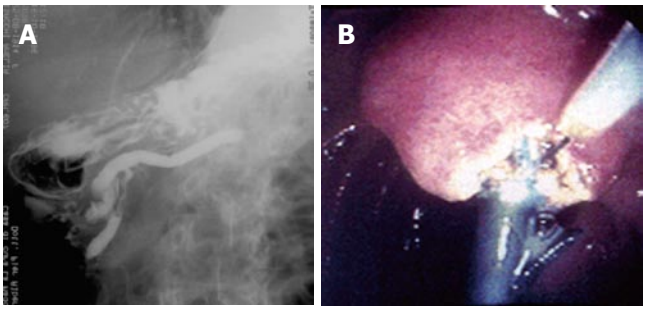

Figure 8 Chronic obstructive pancreatitis in incomplete pancreas divisum. **A**: Minor papilla stenosis associated with diffuse upstream dilation of the pancreatic duct and normal parietal morphology; **B**: Minor papilla sphincterotomy over-the-stent performed with a needle-knife sphincterotome. Minor papilla sphincterotomy rather than long-term stenting appears an appropriate approach in this case with stricture located at the level of the minor papilla.

PANCREAS DIVISUM

Pancreas divisum is present in about 7% of the population; it occurs when the ventral and dorsal ducts of the gland fail to fuse during embryological development. This anatomical variant is asymptomatic in the majority of cases but in some cases it may cause pancreatic pain due to functional obstruction at the level of the minor papilla or recurrent episodes of acute pancreatitis; persistence of the obstruction over time may lead to chronic obstructive pancreatitis. Kamisawa *et al* reported acute recurrent pancreatitis and chronic pancreatitis associated with pancreas divisum in respectively 17.1% and 28.6% of their patients[22].

Endoscopic therapy with minor papilla sphincterotomy and/or stent placement appears to be the treatment of choice at present. Critical issues concerning endotherapy in pancreas divisum are patient selection, difficulty of papillary cannulation, technique for endotherapy (minor papilla sphincterotomy or dorsal duct stenting, or both), stent-induced duct injury, and risks of post-ERCP pancreatitis. Patients with acute recurrent pancreatitis are the best candidates for endotherapy as in this group the predicted sustained response rate is around 75%; the response rate in patients with chronic pancreatitis is 40%-60%, whereas patients with recurrent or chronic abdominal pain respond poorly (20%-40%)[23].

The minor papilla is often difficult to visualize, but its orifice can be easily identified by spraying methylene blue over the duodenal mucosa in the papillary area or injecting it directly into the ventral duct, in cases with incomplete pancreas divisum[24], or by EUS[25], or by enhancing pancreatic secretion with i.v. secretin[26].

Endotheraphy of pancreas divisum includes minor papilla sphincterotomy and dorsal duct stenting with 5F, 7F and 10F stents, depending on the level of obstruction and degree of dilation (Figure 7 and Figure 8). Dorsal duct stenting without sphincterotomy was adopted by McCarthy *et al*[27], Lans *et al*[28] and Ertan[29], who reported satisfactory long-term results in respectively 89%, 90% and 76% of cases. However, Heyries *et al* reported more favorable long-term results with minor papilla sphincterotomy than with stenting[30]; they also observed fewer complications after sphincterotomy (25%) than after stenting (44%). More recently however, 45% of patients with chronic pancreatitis associated with pancreas divisum, undergone successful dorsal duct stenting and followed for a median period of 59.6 mo, required surgery after stent removal because of recurrence of symptoms[31]. Of course, stenting is the only option in cases with dorsal duct strictures proximal to the papillary orifice. A strategy of empiric 3-6 mo dorsal duct stenting may be adopted in patients with recurrent pain or pancreatitis with non-dilated dorsal duct or normal minor papilla motor function, investigated by manometry or MRCP and secretin test, in order to decide whether sphincterotomy would be appropriate. This approach in patients with non-pathological duct morphology, however, could lead to ductal changes consistent with chronic pancreatitis in about one third of cases.

PANCREATIC PSEUDOCYST WITH DUCTAL COMMUNICATION

Pseudocysts complicate acute and chronic pancreatitis in up to 20% of cases; approximately 50% of pseudocysts regress spontaneously within 6 to 12 wk. Pseudocysts that are symptomatic, or become larger on follow-up imaging, or are associated with complications, require a drainage procedure. The pseudocyst communicates directly with the MPD in up to 40%-66% of cases[32]. Pseudocysts with ductal communication can only be resolved by duct drainage[33]. This can be achieved during ERCP by a trans-papillary approach, thus avoiding the usual risks (bleeding and perforation) of endoscopic cysto-gastrostomy or cysto-duodenostomy, especially when endoscopic ultrasound (EUS) guidance is not available. Trans-papillary 5F, 7F or 10F stents can be placed beyond the strictured segment of the MPD but not into the pseudocyst in cases with duct strictures downstream of the pseudocyst, or

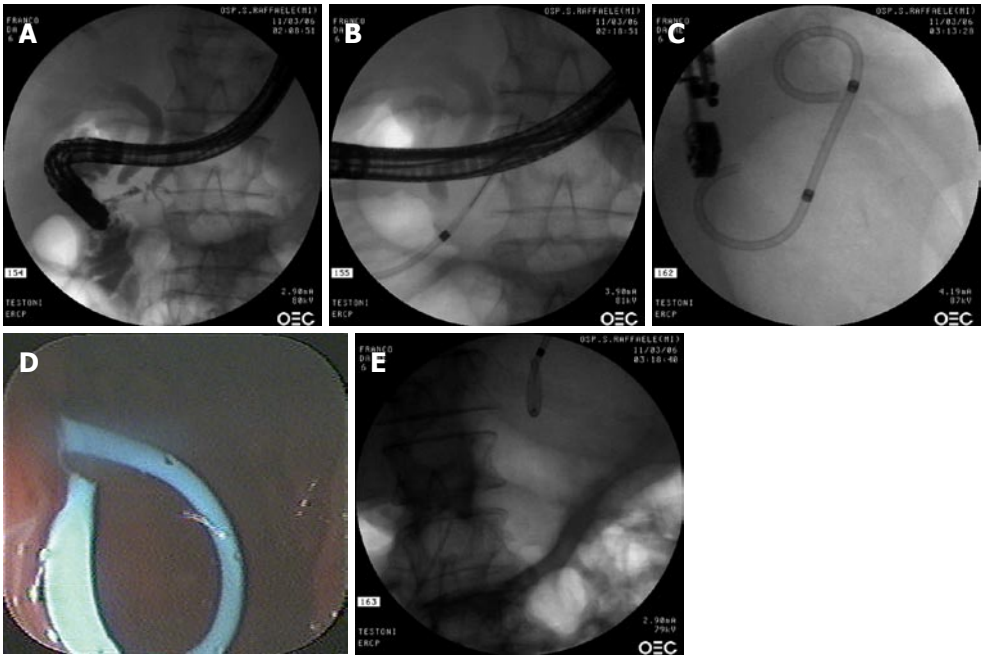

Figure 9 Large pancreatic pseudocyst communicating with the main pancreatic duct: combined endoscopic trans-papillary and trans-gastric drainage. **A**: Contrast injection into the MPD shows a stricture of the main pancreatic duct at the level of the cyst; **B**: After pancreatic sphincterotomy, the stricture is dilated, a guidewire is inserted into the cyst cavity, and a plastic stent is placed; **C** and **D**: Under EUS guidance, a double pig-tail plastic stent is also placed by a single step procedure; **D**: X-ray imaging shows trans-papillary and trans-gastric stents at the end of the procedure.

Table 1 Endoscopic transmural drainage of pancreatic pseudocyst[1]

Endoscopic centers	198
EUS-guided drainage	65%
Successful drainage	91%
Clinical success	71%
Mean number of stents inserted	2
Mean duration of stenting	6 wk
Complications rate	
Infection	12.70%
Bleeding	11.40%
Perforation	2.20%

[1]: International survey of ASGE members.

directly into the pseudocyst cavity if no MPD strictures are found[34], or into the MPD bridging the communication between the duct and the cyst cavity. When the stent is placed directly in the pseudocyst cavity, a double pig-tail stent should be preferred to avoid the risk of displacement. In the presence of a large and symptomatic pseudocyst, MPD drainage is generally associated with a trans-parietal drainage in order to achieve a more rapid decompression of the cyst cavity and resolution of symptoms (Figure 9). EUS-guided pseudocyst drainage has become popular in recent years because it has many advantages compared to the endoscopic approach[35]. Bulging is not required; ultrasound guidance permits assessment of the optimal area to access vascular structures, cyst content and communication with the main pancreatic duct (Table 1). Pseudocyst drainage is feasible even if the distance between the cyst and G.I. lumen exceeds one cm, and the procedure may be performed in a single step.

Stents should be changed routinely every 6-8 wk, to avoid clogging and the risk of infection or pancreatitis.

Features predictive of a successful trans-papillary approach are MPD dilation upstream of the ductal stricture when the stent is placed across the stricture and a non-dilated pancreatic duct when the stent is placed to bridge the communication of the cyst with the pancreatic duct. Placing a stent in the pseudocyst in a case with non-dilated MPD is associated with a higher risk of pancreatitis.

PANCREATIC FISTULAS

Fistulas can occur as a consequence of partial or complete rupture of the pancreatic duct caused by trauma, pancreatic surgery, or complicating severe acute pancreatitis. During an attack of acute severe pancreatitis, ERCP found a pancreatic duct leak in 37% of cases and this was significantly associated with a higher incidence of necrosis and longer hospital stay[36]; the early recognition and treatment of such leaks and eventually associated fluid collections is likely to improve outcomes[37].

At present, the diagnostic approach to pancreatic fistulas and suspected pancreatic duct leaks should be MRCP with secretin stimulation, leaving ERCP and EUS only for therapeutic purposes, once the lesion has been identified and staged. Before planning endoscopic treatment of fistulas or duct leaks several points must be clarified: the location of the lesion within the pancreatic ductal system, the presence and type of pancreatic duct strictures downstream of the lesion, whether pancreatic duct disruption is complete or incomplete, whether there is any communication with a fluid collection, and its anatomical characteristics.

Fifteen years ago Kozarek *et al* reported that bridging a pancreatic duct leakage by trans-papillary stent placement was effective for either internal or external pancreatic fistulas[38]. Transpapillary stenting of the MPD has now become the "gold standard" for the treatment of fistulas and duct leaks, with success rates ranging from 55%[39] to 100%[40], although higher than 80% in most series (Figure 10 and Figure 11). Telford *et al* reported that the position of the bridging stent was the only variable related with a good outcome (92%), while stents placed at the level of

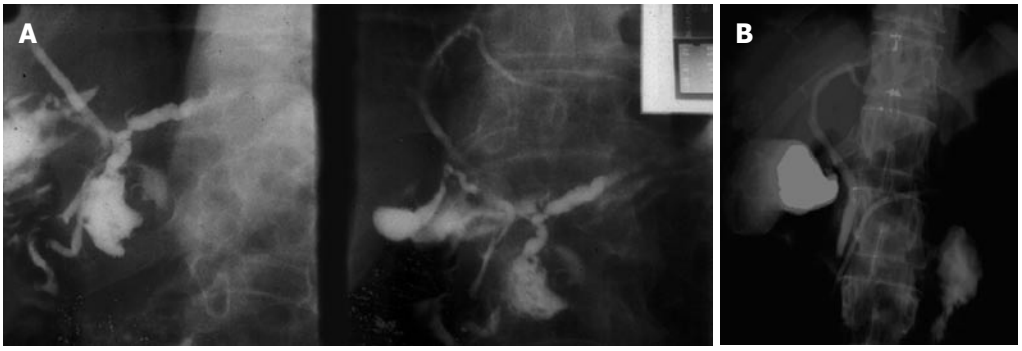

Figure 10 Main pancreatic duct disruption with pancreatic juice leakage (**A**) successfully treated by plastic stent insertion bridging the leak (**B**).

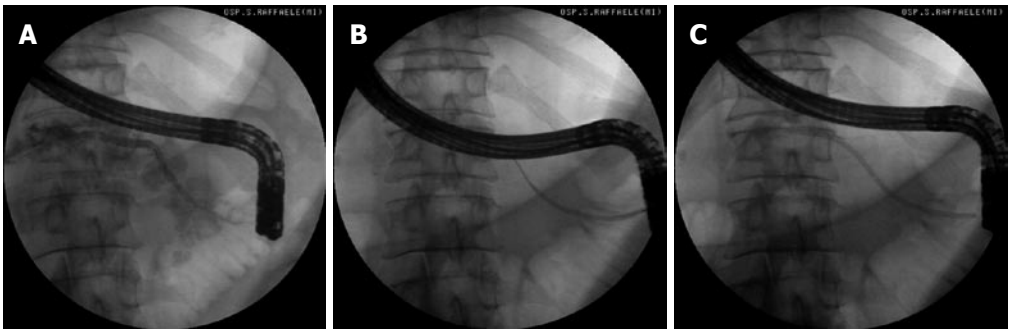

Figure 11 Pancreatic duct leakage at the level of the tail of the gland, following surgical resection of neuroendocrine tumor. **A**: The leakage site is identified by contrast injection into the main pancreatic duct; **B** and **C**: Over a guidewire, a long plastic stent is inserted at the level of the tail of the gland.

the leakage or distally were more often associated with approximately 50% of failures[41]. A partially disrupted MPD, the location of the disruption at the level of the body of the pancreas, the stent positioned to bridge the disruption, and a longer duration of stent therapy were identified as predictors of a favorable outcome in the endoscopic management of duct disruption on a large series of patients[39]. The stent should be left in place for four to six weeks. A shorter period of stenting may involve a higher rate of failure[41], while a longer period may increase the risk of stent occlusion and stent-induced alterations in ductal morphology.

SMOLDERING PANCREATITIS AND IDIOPATHIC RECURRENT PANCREATITIS

"Smoldering" pancreatitis refers to a syndrome in which patients recovering from acute pancreatitis suffer from unremitting abdominal pain, intolerance of food, persistently elevated serum levels of pancreatic enzymes, and persisting inflammatory changes in and around the pancreas at imaging studies. Functional obstruction of the papillary orifice, induced by edema or sphincter spasm, and inflammation-related fibrotic strictures of the MPD may account for the unremitting course in a subset of patients with smoldering pancreatitis. In these cases, insertion of a stent into the MPD provided permanent relief of pain in 91% of patients within a mean of nine days (range 3-20 d) and discontinuation of parenteral nutrition within a mean of 15 d (range 7-39 d); the stents were left in place for a mean of seven weeks (range 2-19 wk)[42].

Today the etiology of acute pancreatitis remains undefined in 2%-30% of cases, despite a careful diagnostic work-up including imaging techniques for pancreatic morphology (CT scan, MRCP, EUS), functional investigation of the sphincter of Oddi (manometry, secretin test), and tests for gene mutations and autoimmune disorders. In these cases the term "idiopathic pancreatitis" is generally adopted and the failure to identify the cause predisposes to further recurrences. Despite the absence of morphofunctional alterations, however, it is generally believed that biliary sludge or microlithiasis or unrecognized transient sphincter of Oddi dysfunction (Type 2) plays a causal role. In a therapeutic protocol study adopted in our institution we found that the placement of a 5F or 7F stent into the MPD in cases with pancreatitis still recurring after biliary sphincterotomy served to identify those patients with residual hypertension of the pancreatic segment of the sphincter of Oddi who benefit from pancreatic sphincterotomy, as documented during a 27-mo follow-up. In these patients with a non-dilated MPD stents were routinely changed every three months. This empiric approach can be suggested for patients with recurrent pancreatitis but no evidence of morphofunctional abnormalities, presenting at least two or three acute attacks over one year, in whom three to six months' stenting can provide a reliable basis for deciding on pancreatic sphincterotomy[43].

Jacob *et al*[44] reported the results of a prospective randomized nonblinded trial evaluating the effectiveness of pancreatic stent placement in preventing attacks of pancreatitis in patients with idiopathic recurrent pancreatitis over a five-year period. The stent group received three stents in one year while the control group underwent selective pancreatic duct opacification without stenting. Pancreatitis recurred in 53% of the control group and in 11% in the stent group. The authors concluded that unrecognized intermittent pancreatic duct sphincter dysfunction or relative outlet obstruction might be a cause of recurrent pancreatitis that can be prevented by stent

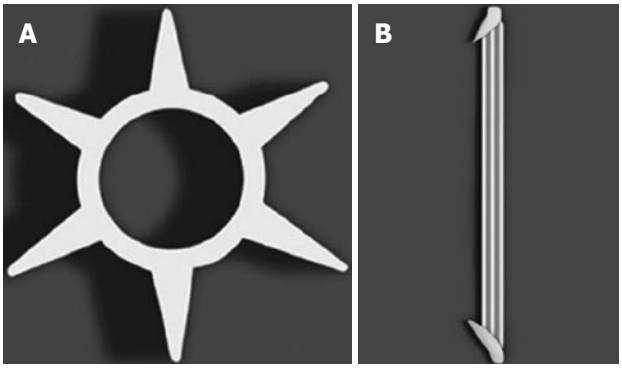

Figure 12 Experimental winged 5 and 7F stents. The new stent design with a wing shape permits an adequate flow of pancreatic juice even alongside the stent and does not compress the duct over its entire circumference, thus avoiding the risk of impaired drainage of pancreatic juice and mechanical trauma to the duct.

placement. However, long-term stenting of the pancreatic duct may in itself cause ductal damage, so only short-term stenting in patients with frequent episodes of pancreatitis is justifiable.

COMPLICATIONS OF STENT PLACEMENT

Several complications have been reported after pancreatic duct stent placement in benign diseases, ranging from 5%-39%. These include inward or outward migration of the stent, occlusion, and anatomic changes of the pancreatic duct[45,46]. The latter limits the long-term use of stents in the treatment of benign disorders especially when pancreatic ducts are non-dilated. Changes of MPD morphology consistent with chronic pancreatitis have been reported after stent placement in 36%-83% of patients; ductal changes of the pancreatogram appear as early as three months and seem not to revert to normal in some cases after removal of the stent. Pancreatic stents placed in dogs were found to induce both radiological and histological changes of chronic pancreatitis in the ductal segment treated with the stent, within eight weeks[47].

Although the mechanism by which changes are induced is not known, there is evidence that stenting the pancreatic duct leads to the formation of intraductal plugs in as little as three weeks even though pancreatograms may remain normal. These protein precipitates have the same composition as plugs removed from patients with chronic pancreatitis. Moreover, the conventional plastic stent does not provide enough side openings for unencumbered drainage at all sites where secondary ducts join the MPD; this obstruction and the pancreatic duct compression along the whole length of the stent induce a fibrotic reaction. A new pancreatic stent design with a wing shape has now been tested in dogs, with encouraging results, since this model permits an adequate flow of pancreatic juice even alongside the stent and does not compress the duct over its entire circumference, thus avoiding the risk of impaired drainage of pancreatic juice and mechanical trauma to the duct[48] (Figure 12).

In conclusion, in about the last 20 years endotherapy of pancreatic disorders has evolved from an experimental approach for some pathological conditions in selected cases where there is a fear of severe complications, to the "gold standard" for most acute and chronic inflammatory disorders involving the gland.

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REVIEW

Angiogenesis and vascular malformations: Antiangiogenic drugs for treatment of gastrointestinal bleeding

Juergen Bauditz, Herbert Lochs

Juergen Bauditz, Herbert Lochs, Charité University Hospital, 4th Department of Medicine, Berlin, Germany
Correspondence to: Dr. Juergen Bauditz, Universitätsklinikum Charité, IV. Medizinische Klinik und Poliklinik, Charitéplatz 1, Berlin 10117, Germany. juergen.bauditz@charite.de
Telephone: +49-30-450614277 Fax: +49-30-450514906
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Abstract

Treatment of gastrointestinal bleeding in patients with angiodysplasias and Osler's disease (hereditary hemorrhagic telangiectasia) is clinically challenging. Frequently, vascular malformations occur as multiple disseminated lesions, making local treatment an unfavorable choice or impossible. After local therapy, lesions often recur at other sites of the intestine. However, as there are few therapeutic alternatives, repeated endoscopic coagulations or surgical resections are still performed to prevent recurrent bleeding. Hormonal therapy has been employed for more than 50 years but has recently been shown to be ineffective. Therefore, new therapeutic strategies are required. Understanding of the pathophysiology of angiogenesis and vascular malformations has recently substantially increased. Currently, multiple inhibitors of angiogenesis are under development for treatment of malignant diseases. Experimental and clinical data suggest that antiangiogenic substances, which were originally developed for treatment of malignant diseases, may also represent long-awaited specific drugs for the treatment of vascular malformations. However, antiangiogenics display significantly different actions and side-effects. Although antiangiogenics like thalidomide seem to inhibit gastrointestinal bleeding, other substances like bevacizumab can cause mucosal bleeding. Therefore differential and cautious evaluation of this therapeutic strategy is necessary.

Key words: Angiodysplasias; Osler's disease; Angiogenesis; Gastrointestinal bleeding; Vascular endothelial growth factor

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INTRODUCTION

Diseases related to vascular malformation and pathologic vessel growth include benign conditions such as pediatric hemangioma, cutaneous angiectasias and venous malformations. They also include more severe and potentially life-threatening diseases like gastrointestinal angiodysplasias, Osler's disease (hereditary hemorrhagic telangiectasia, HHT) and cerebral arteriovenous malformations, as well as rare syndromes like hereditary dysembryoplasia and pulmonary capillary haemangiomatosis. Vascular malformations and dysfunctional vessels in these diseases result from different disturbances of the angiogenic process, which have only been partially characterized[1-3].

Among these anomalies, arteriovenous vascular malformations that are located on mucosal surfaces are of special clinical relevance because such lesions may cause intense bleeding. Other symptoms of arteriovenous vascular malformation include shunt syndromes, compression syndromes and thrombocytopenia/coagulopathy (Kasabach-Merritt syndrome).

Both Osler's disease and gastrointestinal angiodysplasias can cause recurrent bleeding, which in severe cases can require hundreds of blood transfusions over a period of years[4,5]. Despite diagnostic improvements like wireless capsule endoscopy, treatment of such patients remains a clinical challenge. Multiple lesions disseminated over the small intestine are frequently present, making local treatment an unfavorable choice or impossible[4]. However, as there are few therapeutic alternatives, repeated endoscopic coagulation or surgical resection is performed to prevent recurrent bleeding (although lesions often recur at other intestinal sites after local therapy). Therefore an effective medical treatment for these patients is urgently needed.

Although multiple drugs have been evaluated, there is currently no medical treatment with confirmed efficacy for preventing bleeding from vascular malformations. In this situation, hormonal therapy, which is still the best evaluated treatment option, is frequently employed. Based on observations of cycle-dependent severity of bleeding in some patients, hormonal therapy with estrogens and progesterones has been used for prevention of bleeding since the 1950s. However, there have been no confirming data regarding the effectiveness of hormonal therapy for prevention of intestinal bleeding. The only double-blind, placebo-controlled trial for treatment of nasal bleeding in Osler's disease suggested a moderate effect, but found no

significant reduction in epistaxis from estradiol therapy[6]. In 72 patients with non-hereditary angiodysplasias, a recent randomized placebo-controlled study failed to show an effect from a combination of ethinyl estradiol and norethisterone on the incidence of rebleeding and transfusion requirements[7]. Due to these disappointing results and substantial side-effects, especially in male patients, new therapeutic approaches are clearly required.

MECHANISMS OF BLEEDING

Understanding of the pathophysiology of angiogenesis and vascular malformation has recently substantially increased. Different mutations within the angiogenic signaling cascade indicate that two types of Osler's disease can be differentiated. Mutations of endoglin or activin receptor-like kinase 1 (ALK-1), both receptors for transforming growth factor beta (TGFβ)[1,2], are present in the majority of cases of HHT. Differential stimulation of ALK-1 and ALK-5 is thought to regulate different phases of angiogenesis, thereby activating the angiogenic process, with subsequent increased production of vascular endothelial growth factor [VEGF, initially termed vascular permeability factor (VPF)][8,9]. VEGF, so far the best characterized angiogenic molecule, stimulates proliferation of vascular endothelial cells and increases vascular permeability, and thus is a central mediator of the early phase of angiogenesis[10]. Recent studies have confirmed that both Osler's disease and nonhereditary intestinal angiodysplasias are characterized by increased production of VEGF[11,12]. High serum levels of VEGF that also correlate with severity of bleeding are found in patients with Osler's disease[11].

Intestinal angiodysplasias in patients who have undergone colonic resections because of recurrent bleeding strongly express VEGF along the endothelial lining[12], indicating a proliferative phase of angiogenesis. Expression of VEGF receptor 1 is also observed in bleeding intestinal angiodysplasias[12]. As VEGF receptor 1 is specifically up-regulated by hypoxia, this finding may indicate a role for hypoxia in the pathogenesis of angiodysplasias[13,14]. However, further detailed analyses are necessary to clarify the pathophysiology of gastrointestinal angiodysplasias.

VEGF-mediated effects are presumably also involved in bleeding in inflammatory bowel disease (IBD). The mucosal inflammation in Crohn's disease and ulcerative colitis is characterized by increased production of the proinflammatory cytokines tumor necrosis factor α, interleukin (IL)-1 and IL-6. In experimental models, proinflammatory cytokines have been shown to induce VEGF[15,16], a mechanism which is also likely present in IBD in order to supply the inflammatory infiltrate and thickened bowel wall with microvessels[17]. As a result of permanent VEGF stimulation, sprouting vulnerable vessels are located at the inflamed mucosal surface; thus possibly resulting in intermittent bleeding and so contributing to chronic anemia in Crohn's disease[18,19]. However, a small number of patients with Crohn's disease also suffers from massive recurrent bleeding[20,21]. As such episodes of severe bleeding are not associated with high disease activity[20-22], it can be speculated that the severe bleeding in such patients results from larger superficially located vessels with arteriovenous short circuits. Angiodysplasias have been documented in several of these patients; however they have not been definitely proven to cause this bleeding[20-22].

The initial phase of angiogenesis is characterized by VEGF-dependent formation and the sprouting of vessels consisting of endothelial cells. Within this process, microenvironmental concentrations of VEGF seem to determine whether normal or aberrant angiogenesis is induced[23]. Gene-therapy-induced high local concentrations of VEGF result in vascular malformations[24]. Such lesions resemble the chaotic architecture of haemangiomas or angiodysplasias and are characterized by thin-walled fragile vessels with high permeability, which lack smooth muscle cells and are susceptible to rupture[25]. To mature, such a primitive vascular plexus would have to be remodeled and vessels acquire a smooth muscle layer, a process that requires other angiogenic factors such as TGFβ, platelet-derived growth factor and angiopoietin-1 (which also stabilizes the leakage of VEGF-overexpressing vessels)[25,26]. Angiodysplasias and other vascular malformations like hemangiomas arise from massive local activation of the early stage of angiogenesis, with accumulation of VEGF, which induces primitive endothelial vessel complexes. However, these vessel precursors fail to finally differentiate into complete functional vessels and form net-like labyrinthine complexes. If surrounded by parenchymal tissue or stable epithelial structures, these complexes are generally harmless. However, when located near a mucosal surface, such fragile vessel systems are susceptible to rupture and can cause bleeding.

ANTIANGIOGENIC THERAPY

The observation that different vascular malformations, despite a distinct pathogenesis, are characterized by a pathologic accumulation of VEGF theoretically makes them an attractive target for direct or indirect VEGF-suppressive antiangiogenic therapy. Suppression of VEGF disrupts development of sprouting vessels: in experimental models VEGF withdrawal results in endothelial cell shedding and regression of primitive hemangioblastoma-like vessels[27].

Several antiangiogenic substances have been developed for treatment of malignant diseases. These include monoclonal antibodies against VEGF [Bevacizumab (Avastatin)], VEGF-trap, VEGF-receptor antibodies and antagonists [SU5416 (semaxanib), IMC-IC11, PTK 787 and SU6668], proteins (endostatin and angiostatin), matrix metalloproteinase inhibitors (Marimastat, Primostat and COL-3), thalidomide and its analogues [CC5013 (Revimid) and CC4047 (Actimid)], and several other substances that act during different phases of the angiogenic process[28]. In addition to their therapeutic potential in malignant diseases, some of these substances could also be useful for treatment of bleeding vascular malformations.

THALIDOMIDE-THE FIRST ANTIANGIOGENIC DRUG

The best known-and for four decades unrecognized as

such-inhibitor of angiogenesis is thalidomide, which tragically was used as a sedative and anti-emetic in pregnant women from 1956 until it was withdrawn from the market in 1961 after being recognized to have caused severe birth defects. During these years, in which thalidomide became the most popular sedative in Germany, about 10000 children with phocomelia and other malformations were born[29]. It was only in 1994 that thalidomide was found to inhibit VEGF-and basic fibroblast growth factor-mediated angiogenesis; a detection that resulted from a side-effect-based literature screening in search of drugs with antiangiogenic activity that D'Amato and Folkman presumed should cause both amenorrhea and fetal malformations[30]. The exact mechanism by which thalidomide acts within the angiogenic cascade remains to be determined, but appears to be located upstream of the VEGF level, i.e. reduced expression of integrin genes has been hypothesized[31]. In experimental models, the antiangiogenic activity of various thalidomide metabolites correlates with teratogenicity[30-32], indicating that antiangiogenic effects are also mainly responsible for thalidomide-related birth defects in humans. The antiangiogenic potential of thalidomide is currently being evaluated for treatment of several malignant diseases[29]. Due to promising results in patients with therapy-refractory multiple myeloma, thalidomide is now evaluated as both first-line myeloma therapy and in combination with hematopoietic-cell transplantation[33,34].

It has recently been reported in a number of case studies that thalidomide reduces the incidence of severe bleeding in different gastrointestinal diseases. Eight patients with severe bleeding related to Crohn's disease or angiodysplasias of the small intestine, who had received up to 230 blood transfusions, responded to moderate doses of 100-300 mg thalidomide daily[18,22,35]. Similarly, chronic bleeding unexpectedly resolved in patients with hereditary hemorrhagic telangiectasia, who received thalidomide as antiangiogenic cancer therapy[36,37]. In patients with angiodysplasias, rebleeding was also prevented for more than 2 years after thalidomide treatment had ended[22]. In Crohn's disease with moderate inflammatory activity, severe bleeding stopped during thalidomide treatment, partly recurred after cessation of thalidomide, but was controlled again by retreatment[18,22]. However, in patients with Crohn's disease, it is not clear whether cessation of bleeding is related to antiangiogenic or anti-inflammatory effects of thalidomide. More objective evidence that antiangiogenic effects of thalidomide are responsible for the efficacy on bleeding is found in patients with non-inflammatory diseases. In patients with angiodysplasias or Osler's disease without any evidence of inflammation[22,35,37], the efficacy of thalidomide on bleeding is unlikely to be related to anti-inflammatory or immunomodulating effects. Serum levels of VEGF were found to be suppressed by thalidomide in patients without inflammation[22]. In patients with multiple angiodysplasias of the small bowel, wireless capsule endoscopy has demonstrated that the clinical efficacy of thalidomide is paralleled by a decrease in number, size and color intensity of angiodysplasias, which indicates regression[38].

Although the results of the above case series need to be confirmed in controlled trials, present data indicate that antiangiogenic effects of thalidomide are responsible for reductions in bleeding episodes. However, the side effects of thalidomide are also substantial. Thalidomide is a potent sedative, causes severe birth defects and can also induce sensible peripheral neuropathy, especially at higher cumulative doses[39], therefore, it may not turn out to be the drug for treatment of vascular malformations hoped for by clinicians. Furthermore, in addition to its antiangiogenic activity, thalidomide exerts immunomodulating effects[29]. It is possible that other newly developed antiangiogenics will show more specific inhibition of VEGF or other steps within the angiogenic cascade, with possibly fewer side effects.

NEW ANTIANGIOGENICS

Of the currently developed antiangiogenic substances, most information regarding clinical efficacy and toxicity is available for bevacizumab (Avastatin®), a humanized monoclonal antibody against VEGF. Bevacizumab was recently shown to be effective in the treatment of colonic and renal cancer; present data indicate strong antiangiogenic activity and a favorable side-effect profile[40,41]. However, nose bleeding is frequently observed during treatment (in up to 59% of patients)[42]. The incidence of nose bleeding correlates with higher doses[40], although the reason for this bleeding is not clear. A loss of vascular integrity by bevacizumab-induced endothelial-cell shedding in highly regenerative mucosal tissues with active angiogenesis could be a possible explanation for this dose-dependent effect. Other reported side effects during treatment with bevacizumab include gastrointestinal bleeding and perforations, which were not always tumor-related[41,42].

Bleeding complications do not seem to be a specific feature of bevacizumab. A recent study with IMC-IC11, a humanized monoclonal antibody against VEGF receptor 2, also reported bleeding episodes unrelated to tumor manifestations[43]. Although most bleeding complications are probably of minor relevance in patients with malignant diseases, it is conceivable that abrupt antibody-induced withdrawal of VEGF in proliferating VEGF-dependent endothelial vessels could become critical in pre-existing vascular malformations located on mucosal surfaces.

Therefore, although VEGF-based antiangiogenic therapy is a promising and highly specific therapeutic option for preventing growth of vascular malformations, it seems questionable whether monoclonal antibodies against VEGF or its receptors are also useful for treatment of bleeding from pre-existing vascular malformations. Indeed, some highly effective antiangiogenics could even aggravate bleeding from vascular malformations.

Semaxanib (SU 5416), a small-molecule inhibitor of VEGF receptor 2 tyrosine kinase has recently been used for treatment of hemangioblastoma in patients with von Hippel-Lindau disease (vHLD). In vHLD, a loss of von Hippel-Lindau protein results in an accumulation of hypoxia-inducible factor and subsequently, induction of

VEGF[44]. Some initial studies on semaxanib have reported regression or stabilization, with improvement of macular edema, in patients with hemangioblastoma, which indicates effective inhibition of VEGF[44,45]. Frequently observed side effects of semaxanib include fatigue and headache[47,48]. To date, bleeding complications, like those found for bevacizumab, have not been reported for semaxanib (neither have they been reported for thalidomide, for which side effects have been well documented since its reevaluation for malignant and inflammatory diseases).

In summary, it is remains unclear why some antiangiogenic substances like bevacizumab can cause mucosal bleeding and others like thalidomide do not. This effect may be related to the phase of angiogenesis that is antagonized, or might reflect a particular strong antiangiogenic activity. Detailed analyses of the angiogenic cascade and how thalidomide and other antiangiogenics act within this process will be needed to resolve this issue.

SIDE-EFFECTS AND TOXICITY

Currently, only limited data are available regarding the general toxicity of antiangiogenic therapy. Arterial hypertension has been reported for several agents and is thought to be at least partially related to reduction of VEGF-mediated vascular permeability. As VEGF is also centrally involved in neuroregeneration, neurotoxicity is another possible concern for antiangiogenic treatment[49]. Experimental peripheral neuropathy is reversible by VEGF gene transfer[50]. Reduction of VEGF levels by 25% results in motor neuron degeneration reminiscent of amyotrophic lateral sclerosis[51]. Thalidomide's well-documented neurotoxicity is therefore possibly not drug-specific but could be a general effect of long-term antiangiogenic therapy. Until now, significant neuropathy has not been reported for the new antiangiogenics; however, only very limited data regarding long-term toxicity are available. Furthermore, as these drugs are generally just one part of a polychemotherapeutic regimen, it is often unclear to what extent antiangiogenics contribute to clinically observed neurotoxicity[52].

Finally, VEGF is also crucial for embryonal angio- and vasculogenesis. Loss of a single VEGF allele causes severe embryonic vascular defects[53]. Therefore, not only thalidomide, but any inhibitor of VEGF that crosses the placenta has to be considered a potential teratogen. As antiangiogenics are primarily developed as anticancer agents and designated to be used in combination with chemotherapy, this subject is generally regarded as of minor relevance. However, if antiangiogenic therapy is expanded to benign diseases like angiodysplasias, Osler's disease, and inflammatory diseases like Crohn's disease (which may also improve due to inhibition of angiogenesis)[54-57], and young women are candidates for treatment, teratogenicity becomes a critical issue. Indeed, a single dose of thalidomide is sufficient to cause birth defects[30]. Angiogenesis has a central role in embryo growth and pregnancy-prevention programs cannot completely prevent the birth of children with fetal malformations[58]; therefore, antiangiogenics should only be used under strict surveillance in non-malignant diseases.

CONCLUSION

In summary, antiangiogenic substances like thalidomide hold promise to be not only useful for treatment of malignant diseases, but may also represent the drugs that have been long awaited for the treatment of bleeding from vascular malformations. However, as some antiangiogenics can cause mucosal bleeding, a differential therapeutic approach and careful evaluation are necessary. Moreover, antiangiogenic therapy is also teratogenic and therefore has to be very cautiously considered in women of child-bearing potential.

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REVIEW

Updates on abdominal desmoid tumors

Bernardino Rampone, Corrado Pedrazzani, Daniele Marrelli, Enrico Pinto, Franco Roviello

Bernardino Rampone, Corrado Pedrazzani, Daniele Marrelli, Enrico Pinto, Franco Roviello, Department of General Surgery and Surgical Oncology, Unit of Surgical Oncology, University of Siena, Italy
Correspondence to: Dr. Bernardino Rampone, Department of General Surgery and Surgical Oncology, Unit of Surgical Oncology, University of Siena, Policlinico Le Scotte, viale Bracci, 1, 53100, Siena, Italy. ramponebernardino@virgilio.it
Telephone: +39-5-77585156 Fax: +39-5-77233337
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Abstract

Desmoid tumor is a monoclonal, fibroblastic proliferation arising in musculoaponeurotic structures. This connective tissue hyperplasia infiltrates locally, recurs frequently after resection but does not metastasize. Abdominal desmoid occurs sporadically, in association with some familial syndromes and often represents a clinical dilemma for surgeons. The enigmatic biology and anatomical location of abdominal desmoids make treatment recommendations difficult. This distinct pathological entity is reviewed with a specific focus on aetiology and management.

Key words: Desmoid; Abdomen; Fibromatosis; Familial adenomatous polyposis; Gardner's syndrome

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INTRODUCTION

Desmoid tumor, also known as aggressive fibromatosis or musculo-aponeurotic fibromatosis[1], is a monoclonal[2,3], fibroblastic proliferation arising in musculoaponeurotic structures.

Although Mueller in 1838 coined the term desmoid tumor[4] (derived from the Greek *desmos* that means tendon-like), the first description of the tumor is credited to McFarlane, who reported the disease occurring in the abdominal wall of a young woman after delivery in 1832[5].

Histologically, these tumors consist of spindle-shaped cells in a collagenous matrix without the pleomorphic, atypical, or hyperchromatic nuclei of malignancy[1]. The connective tissue hyperplasia infiltrates locally, recurs frequently after resection but does not metastasize[6].

Desmoid tumors have been recently subdivided according to their location into extra-abdominal, abdominal and intra-abdominal, and the latter have been subclassified further into mesenteric fibromatosis and pelvic fibromatosis[7]. This tumor may occur at the site of any fascia, but in particular in muscle, hence the descriptive term musculo-aponeurotic fibromatosis. The most frequent sites involved by these tumors are the torso and the extremities. Many studies have shown that between 37% and 50% of desmoids arise in the abdominal region[6,8,9]. Abdominal desmoid occurs sporadically[8], in association with some familial syndromes[9] and often represents a clinical dilemma for surgeons. Most surgical reports emphasize the difficulty in achieving adequate resection margins, while maintaining acceptable function and cosmesis[10,11]. These are major factors contributing to the high rates of relapse after surgery, especially after conservative resections[12,13].

The enigmatic biology and anatomical location of intra-abdominal desmoids make treatment recommendations difficult. A significant factor limiting the attempted generalization concerning management is the small number of cases available for analysis, reflecting the relative rarity of the disease.

EPIDEMIOLOGY AND ETIOLOGY

Desmoid tumor is a rare lesion representing < 3% of all soft tissue tumors with an estimated incidence of 2-4 new cases per million per year[14].

These tumors have been well characterized from a morphologic standpoint, but their nature and pathogenesis have remained obscure for many years, to the point that Stout[15] defined it as "the most incomprehensible group" of fibromatosis. They have been considered non-neoplastic processes by some authors and well-differentiated low-grade sarcomas by others[2].

An association with familial adenomatous polyposis of the colon (FAP) and Gardner's syndrome has been well documented. Abdominal and extra-abdominal desmoids occur more frequently in FAP patients, with an incidence of 3.5%-32%. In the original Gardner kindred the incidence was 29%[9,16].

The etiology of desmoids has not been well defined. Numerous factors are acknowledged to be strongly associated with their development. An antecedent history of trauma to the site of the tumor, often surgical in nature, may be elicited in approximately 25% of cases[17,18].

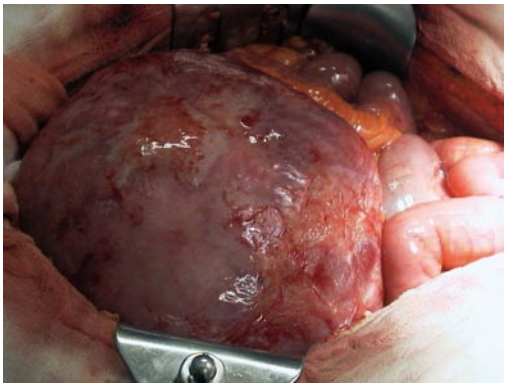

Figure 1 Intraoperative finding of giant retroperitoneal desmoid tumor.

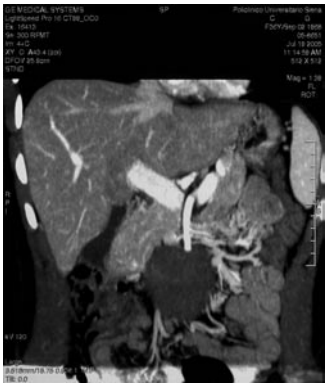

Figure 2 Coronal view of computed tomography scan shows a mesenteric desmoid tumor.

Within the FAP population, there is a strong correlation between prophylactic procto-colectomy and the subsequent development of desmoid tumours[19-22].

Other forms of trauma, such as physiologic trauma associated with pregnancy, are also thought to contribute to the development of desmoid tumors and several papers report an increased association between pregnancy and desmoid tumors[10,14,16,17].

A predominance of the disease in the female population has been reported, with a female to male ratio ranging from 1.4 to 1.8. The peak of incidence is between the ages of 25 and 35 years, even though cases occurring in patients younger than 10 years have been described[10,17,23,24]. The preponderance of cases described afflicting women in reproductive age shows a clear association of this disease with the endogenous hormonal environment and exogenous sex hormones[10,16,17].

Anecdotal reports of tumor regression during menopause[25,26], the development of desmoids in patients taking oral contraceptives[19,27], and reports of tumor regression with tamoxifen treatment[28], serve to underline an evident role of estrogen in the multifactorial pathogenesis of desmoid.

While most of the cases are sporadic, some are associated with familial syndromes (FAP, Gardner's syndrome) and these are most often intra-abdominal[19,29]. There are also cases of familial desmoid tumors at multiple sites, in patients without FAP, often involving one extremity. In both FAP and familial non-FAP tumors, mutations of the adenomatous polyposis coli (APC) gene on the long arm of chromosome 5 have been incriminated. The resultant loss of ability to degrade beta-catenin and elevated beta-catenin levels promotes fibroblastic proliferation through a nuclear mechanism[30].

CLINICAL PRESENTATION AND DIAGNOSTIC EVALUATION

Desmoid tumors most frequently present as a slowly enlarging mass (Figure 1). Symptoms depend on the location of the tumor. Patients with intra-abdominal desmoid may have asymptomatic masses, symptoms of intestinal, vascular and urinary obstruction or neural involvement[6,31,32]. The diagnosis of desmoid is based on clinical suspicion. A history of FAP or of similarly affected family members is frequently elicited. A history of trauma or recent pregnancy is also common.

The role of imaging (computed tomography and magnetic resonance) is to define the degree of extension to local structures and tumor relationship to neurovascular structure[33,34]. This tumor does not metastasize to regional nodes or distant sites so that search for metastatic disease is unnecessary. Biopsy is not usually necessary but does not seem to induce further growth, if performed. At the moment there is no accepted staging system for this disease[35].

MANAGEMENT

The management of desmoid tumors requires special attention. A strong family history of desmoid tumors and a high-risk location of the mutation on the APC gene increase the risk for the development of desmoid tumors[30,36]. Due to a reported 85% recurrence rate of desmoid tumors after surgical excision, surgery should be performed only when absolutely necessary[37,38].

Tamoxifen, toremifene, and sulindac have been used as treatments, but the results are controversial. Reports have suggested that therapy might be associated with an initial benefit but the long-term clinical improvement is minimal[39-41]. Cytotoxic chemotherapy (doxorubicin, dacarbazine, and carboplatin) may be effective in treating aggressive, rapidly growing and unresectable abdominal desmoids[42,43]. Radiation therapy might be effective in selected cases but it is frequently limited by the presence of the small bowel in the radiation field in mesenteric and pelvic desmoids[44,45].

Intra-abdominal desmoid tumors usually involve the mesentery and often involve the mesenteric vessels. They invade the mesentery diffusely, kink loops of bowel and can cause ureters obstruction (Figure 2). This feature requires a complex surgery and often radical resection is impossible to achieve[11]. Therefore the management of intra-abdominal desmoid tumors is complex and is dependant on their clinical behavior.

Given the problems related to the treatment of desmoids, there is a good case for simple observation of many tumors, particularly if asymptomatic. Following diagnosis, a small tumor which is not encroaching on any nearby structures may be followed up by regular clinical

examination (every 6 mo) with or without imaging, usually by CT. Desmoids that are growing slowly or are mildly symptomatic can be treated with sulindac and tamoxifen or with vinblastine and methotrexate, since these are less toxic regimens. Aggressive desmoids are treated with anti-sarcomachemotherapy such as doxorubicin and dacarbazine.

It would thus seem that surgery is a reasonable first-line treatment for abdominal wall tumors, since they are easier to excise than intra-abdominal desmoid tumors, recurrence rates are lower, and morbidity rates associated with the procedure are lower. The excision should be completed with a one cm margin. A mesh can be used to cover the defect if required[46].

For intra-abdominal desmoids surgery should only be used in specific circumstances. These would include tumors which do not appear to involve vital organs and vessels on preoperative imaging, those resistant to drug treatment and in cases where a risky operation is the only possible option in the case of a rapidly growing, life-threatening tumor. High rates of recurrence should be expected and patients must be counseled pre-operatively about the risks of death. Such cases should only be attempted in specialist centers with sufficiently experienced staff.

At the moment one center has also reported a technique where the tumor and small bowel are removed en bloc, perfused and cooled, and the tumor resected on the bench in a bloodless field with subsequent autotransplantation of the small bowel back into the patient[47]. Recently a report has been published where two desmoids (one intra-abdominal) were treated with percutaneous chemical ablation with acetic acid under-radiological guidance[48]. In both cases there was substantial regression of the tumours within a few months.

Unfortunately, despite any treatment, some patients deteriorate, become dependent on TPN, and have life-threatening complications develop. Intestinal transplantation is the only remaining option for these patients[49].

In conclusion the optimal treatment protocol has not yet been established and, in many cases, a multidisciplinary approach including surgery, chemotherapy, and radiation therapy has been employed. The rarity of cases in even major tumor centers has traditionally limited the ability to study this disease. The notion that a specific genotype can predict the development of an aggressive desmoid tumor in a given patient could prove to be valuable in allowing appropriate patient selection for early therapy or even a chemopreventive strategy. Several novel pharmacologic and biologic treatment approaches are actively being developed, although long-term follow-up is needed for their substantiation[50].

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BASIC RESEARCH

Protective effects of medical ozone combined with traditional Chinese medicine against chemically-induced hepatic injury in dogs

Li-Jie Li, Yun-Gao Yang, Zhi-Ling Zhang, Sui-Feng Nie, Ze Li, Feng Li, He-Yu Hua, Yan-Jun Hu, Hong-Shuan Zhang, Ya-Bing Guo

Li-Jie Li, Zhi-Ling Zhang, Sui-Feng Nie, Ze Li, Feng Li, No. 425 Central Hospital of PLA, Sanya 572008, Hainan Province, China
Yun-Gao Yang, He-Yu Hua, Hong-Shuan Zhang, Department of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, Guangdong Province, China
Yan-Jun Hu, Department of Orthopaedics and Traumatology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China
Ya-Bing Guo, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China
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Correspondence to: Associate Professor Ya-Bing Guo, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China. yabing@fimmu.com
Telephone: +86-20-61641947 Fax: +86-20-87719653
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Abstract

AIM: To investigate the protective effect of medical ozone (O_3) combined with Traditional Chinese Medicine (TCM) Yigan Fuzheng Paidu Capsules (YC) against carbon tetrachloride (CCl_4)-induced hepatic injury in dogs.

METHODS: Thirty healthy dogs were divided randomly into five groups (n = 6 in each group), namely control, oleanolic acid tablet (OAT), O_3, YC and O_3 + YC, given either no particular pre-treatment, oral OAT, medical ozone rectal insulfflation every other day, oral YC, or oral YC plus medical ozone rectal insulfflation every other day, respectively, for 30 consecutive days. After pre-treatment, acute hepatic injury was induced in all dogs with a single-dose intraperitoneal injection of CCl_4. General condition and survival time were recorded. The biochemical and hematological indexes of alanine aminotransferase (ALT), aspartate aminotransferase/alanine aminotransferase (AST/ALT), serum total bilirubin (TBIL), prothrombin time (PT), blood ammonia (AMMO), and blood urea nitrogen (BUN) were measured after CCl_4 injection. Hepatic pathological changes were also observed.

RESULTS: Compared to the other four groups, the changes of group O_3 + YC dogs' general conditions (motoricity, mental state, eating, urination and defecation) could be better controlled. In group O_3 + YC the survival rates were higher ($P < 0.05$ *vs* group control). AST/ALT values were kept within a normal level in group O_3 + YC. Hepatic histopathology showed that hepatic injury in group O_3 + YC was less serious than those in the other four groups.

CONCLUSION: Medical ozone combined with TCM YC could exert a protective effect on acute liver injury induced by CCl_4.

Key words: Carbon tetrachloride; Ozone; Traditional Chinese Medicine

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INTRODUCTION

Medical ozone, a mixture gas of ozone and oxygen, has been used for several decades in the therapy of diabetic foot, arthritis, arterial angiemphraxis and ulcerative colitis[1-9]. It has been used in an empirical fashion in recent years for therapy of viral hepatitis. Rectal insufflation with ozone can reduce hepatic and renal ischemia-reperfusion injury[10-15].

Yigan Fuzheng Paidu Capsules (YC), an empirical formula, is a compound preparation of traditional Chinese medicine (TCM) for treating chronic viral hepatitis. It has been demonstrated experimentally that YC can protect the liver and decrease transaminase level, has anti-lipid peroxidation activity, and *ex vivo* antiviral activity.

This study investigated the effect of medical ozone combined with YC affect on carbon tetrachloride (CCl_4)-induced acute liver injury in order to establish a reliable baseline for clinical application.

MATERIALS AND METHODS

Animals

Thirty healthy mongrel dogs aged 1-2 years, weighing 12-15 kg, were used in the experiments. These dogs received regular feeding, were inspected medically, treated with helminthicide, and acclimatized for at least 1 month prior to the experiments in the Experimental Animal Center of Nanfang Hospital, Southern Medical University. The experiments were carried out in accordance with the animal experiment regulations of the university.

Drugs and reagents

YC was manufactured in the Department of Traditional Chinese Medicine, Southern Medical University (Guang-lian-zhi-zi 2004, No. FPGZ150) with Batch No. 050715. Oleanolic acid tablets (OAT) were produced by Nanguo Biological Pharmacy Co., Guangdong Province, China (Guo-yao-zhun-zi No. H44023537) with Batch No. 040701. CCl_4 was produced by the Chemical Plant of Guangzhou with Batch No. 050317, and mixed with the same amount of peanut oil before use.

Equipment

The medical ozone generator (OZONOSAN alpha Plus 1107, Germany) was registered and licensed for medical therapy by SFDA (registration No. 1570177).

Groups and administration

Thirty healthy dogs were divided randomly into five groups (n = 6 in each group): group control, which received no preconditioning treatment; group OAT, treated with oral OAT at 10 mg/d; group O_3, treated with 8.1 mL/kg medical ozone at 20 μg/mL by transrectal insulfflation every other day; group YC, treated with oral YC at 0.2 g/d; and group O_3 + YC, treated with oral YC at 0.2 g/d plus 8.1 mL/kg medical ozone at 20 μg/mL by transrectal insufflation every other day, for a total of 30 consecutive days. After preconditioning treatment, acute hepatic injury was induced in all dogs with a single intraperitoneal injection of CCl_4 mixture at a dose of 0.9 m/kg body weight.

Measurements

The general condition of the dogs was observed before and after treatment, in terms of motor activity, mental state, eating behavior, urination and defecation. The survival time of each dog was measured accurately in hours.

For biochemical and hematological measurements, intravenous blood was sampled pre- and post-treatment at 24 h, 2, 3, 4, 7 and 14 d. All the blood samples were analyzed immediately by the clinical laboratory of Nanfang Hospital for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), prothrombin time (PT), serum ammonia (AMMO), and blood urea nitrogen (BUN).

For histopathology, multiple liver tissues were obtained through ultrasound-guided percutaneous needle biopsy from each dog before and after the experiment. The formalin-fixed, paraffin-embedded liver sections (5 μm) were stained with hematoxylin and eosin (HE) and Gomori silver. The degree of necrosis and inflammatory cell infiltrate was evaluated on a four-point scale (Table 1), using 20 random fields at 100 × and 400 × magnification per slide, by a blinded pathologist (MIF)[16].

Table 1 Histological assessment of drug induced acute hepatitis in dogs

Score	Centrilobular necrosis	Inflammation in centrilobular areas
0	None	None
1	Isolated necrotic hepatocytes or single row of hepatocyte drop-out in perivenular areas	Mild: inflammatory infiltrate affecting < 50% centrilobular areas
2	> 1 and up to 3 rows of perivenular necrotic hepatocytes	Moderate: inflammatory infiltrate affecting > 50% and < 75% centrilobular areas
3	> 3 rows of perivenular necrotic hepatocytes with confluent and/or bridging necrosis	Severe: dense inflammatory infiltrate affecting > 75% centrilobular areas

Statistical analysis

Overall survival was evaluated by actuarial analysis using Kaplan-Meier estimates. Independent samples test for comparison of biochemical and hematological measurements, and one-way ANOVA and LSD test for histological assessment were performed by SPSS 13.0. P-values < 0.05 were considered statistically significant.

RESULTS

General condition of the dogs

Pre-treatment, all 30 dogs were in good condition. Eating behavior, feces and urine were normal. Post CCl_4 treatment, all dogs began to vomit and lost balance immediately. Except for the O_3 + YC group, all the dogs in the other four groups appeared to prefer to stay still alone or were pacing up and down restlessly, while some of them showed poor mental health and appetites, and yellow urine. In group O_3 + YC, there were no differences before and after treatment, except for two dogs with yellow urine.

Survival analysis

Log Rank, Breslow and Tarone-Ware tests were used for comparison of survival rate in the five groups of dogs. All three tests showed that the survival rate of group O_3 + YC was significantly higher than that of the control group (P < 0.05), while there were no significant differences between any other two groups (P > 0.05) (Figure 1).

Biochemical and hematological measurements

Pre-treatment, there were no significant differences (P > 0.05) between the control and the other four groups for any of the measurements (AST/ALT was not being analyzed at the time). After CCl_4 injection, all the measurement index in the five groups except for AST/ALT showed a tendency to increase during the first three days and then gradually fall. There were significant differences (P < 0.05) between groups OAT and O_3, OAT and YC, OAT and O_3 + YC for PT, and between groups

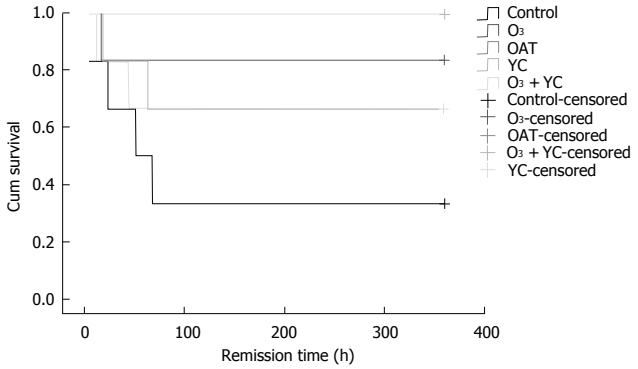


Figure 1 Survival functions of the five groups. The record of each dog's survival time starts at the injection time, and ends in post-360 h (15 d). Plots express the survival rates in different time.

OAT and O_3 + YC, O_3 and O_3 + YC for BUN. In order to compare the measurements post CCl_4 injection, all the index values for each dog were adjusted to the same level. Post CCl_4 injection, the measurement values of ALT, TBIL, PT and AMMO were significantly higher ($P < 0.05$) in the control group than in the other four groups, but there were no significant differences for BUN in all groups ($P > 0.05$). ALT was significantly lower ($P < 0.05$) in group OAT than in group YC, while ALT, TBIL, PT and BUN were significantly higher ($P < 0.05$) in group OAT than in group O_3 + YC. ALT, TBIL, PT and BUN were significantly higher ($P < 0.05$) in group O_3 and YC than in group O_3 + YC. AST/ALT in the control and YC groups showed a little below 1.0 at 24 h after CCl_4 was administrated, then increased to > 1.5 in the control group, and fell to < 0.5 on d 3-4. On d 5-14, AST/ALT in the control group continued to fall (0.1 on d 14). However, in group YC AST/ALT started to increase after 7 d (1.0 on d 14). AST/ALT in the OAT and O_3 groups was approximately 1.0 on first day, fell to approximately 0.4-0.5, and began to increase from d 4 (about 1.0 on d 14) (Table 2).

Liver histopathology

Liver sections from the five groups of dogs, after death or survival for 15 d, were examined and graded for the degree of necrosis and accompanying inflammation, as shown in Table 1. Control group livers demonstrated higher scores for necrosis ($P < 0.05$) and inflammation ($P < 0.05$) compared with the OAT, O_3, YC and O_3 + YC groups (Figure 2).

DISCUSSION

Drug-induced hepatitis is becoming a big problem in clinical practice, and proper management of this problem has not been found so far. For searching new strategy to overcome drug-induced hepatitis, medical ozone and traditional Chinese medicine were first tried in animal model. The effects of medical ozone combined with traditional Chinese medicine on CCl_4-induced acute hepatic injury in dogs were evaluated in this study. The novel method decreased the incidence of jaundice and hepatic encephalopathy and prolonged survival time. Medical ozone combined with YC showed a better effect than OAT, O_3 and YC alone in decreasing transaminases, relieving jaundice and promoting recovery of blood clotting activity.

A number of hepatotoxins have been used to induce fulminant hepatic failure (FHF) in animal models: d-galactosamine, *N*-acetyl-*P*-aminophenol and CCl_4[17,18]. CCl_4 causes serious hepatotoxicity, and has been used extensively and successfully to induce liver damage, including fibrosis[19,20]. CCl_4 is metabolized by cytochrome P450 2E1 in the liver to produce a toxic metabolite. It has been demonstrated to induce liver necrosis, as well as apoptosis[21]. Peroxidation of membrane lipids secondary to the formation of trichloromethyl radicals is believed to be the basis for the toxic effects of CCl_4.

Medical ozone is characterized by its safety and multiple effects in low dose. The main mechanisms of action of ozone pre-conditioning on hepatic injury are as follows. It enhances erythrocyte metabolism[22-26], which can promote the oxygen-carrying capacity of hemoglobin and promote liver oxygenation. Ozone can induce intracellular anti-oxidant enzymes of the liver and scavenging free radicals[14,27-29]. When hepatocytes are equipped with strength against peroxidative radicals by the preconditioning of medical ozone, they can become prone to survival under the attack of toxic CCl_4 radicals. The range of ozone concentrations within which it can exert a therapeutic effect without toxicity is wide from 10 mg/mL to 80 mg/mL[25,30]. YCs have been prescribed clinically for several years. *Astragalus mongholicus*, *Hedyotic diffusa* and *Radix polygoni* prepared as a prescription can promote production of lymphoblasts, regulate the ratio of T-lymphocyte subsets, strengthen immune function, increase activity of lymph active cells and natural killer cells, and induce interferon. Giant knotweed rhizome, *Hedyotic diffusa*, *Rhizome* and *Phyllanthus amatusniruri* have also shown antiviral effects. Honeycomb of paper wasps, red peony root and *Salvia miltiorrhiza* can promote blood flow, mitigate liver injury, and improve hepatocyte oxygenation and promote their recovery. Therefore, the combined effects of O_3 and YC were to up-regulate immune function and promote liver oxygenation. This study may spur the use of a new strategy for the clinical management of some drug-related hepatitis. Clinical trials are needed to confirm the effects of this novel regime.

COMMENTS

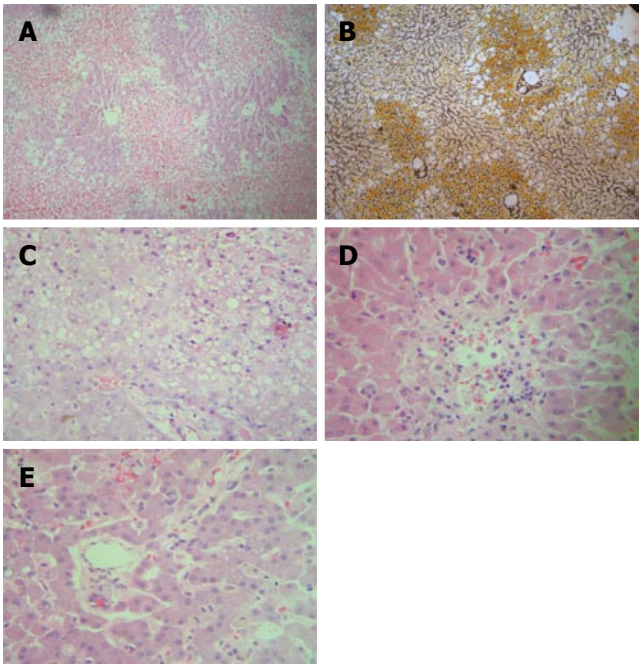
Background

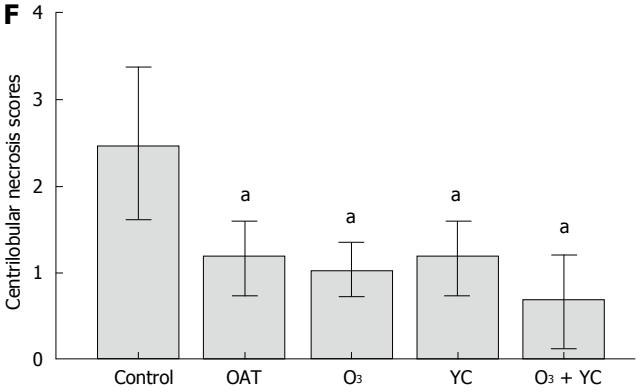
Medical ozone has been wildly used, especially in Europe, in surgery and medicine for 50 years. The technique of ozonetherapy, such as major autohemotherapy, rectal insufflation and topical ozonetherapy, were gradually developed in recent decades and served for more than 10 million patients who suffered from skin ulcerative lesion or trauma, diabetic foot, arthritis, arterial obstruction, ulcerative colitis and spinal disk herniation, *etc*. The property of ozone and its therapeutic effects were partially illuminated. Ozone has opposite faces toward human body. It is harmful gas in high concentration or administration by respiratory tract but therapeutic in low concentration. The milestone fact sheets were that: (1) the ozone reacts with red blood cells (RBC) and reactivates RBC metabolism by increasing 2, 3-DPG and ATP concentration; (2) ozone reacts with immunocompetent cells and induces cytokins release, including IL-2, 4, 10, IFN-β, γ, TGF-β1; (3) ozone activates tissue cells' antioxidative enzymes and enhances capacity of radical scavenger. Each discovery mentioned above will mould scientific base for the novel application scope of ozone. For example, the finding of effect of ozone on immune system is the scientific foundation of using

Table 2 Changes of biochemical and hematological indexes of the five groups (Data represents median ± quartile)

Group	Pre-injection	Post-injection CCl_4					
		Post-24 h	Post-2 d	Post-3 d	Post-4 d	Post-7 d	Post-14 d
ALT/(U/L)							
Control	26.00	1156.00	43.50	1018.00	1790.50	1349.50	297.00
	(16.50-33.75)	(245.00-1641.75)	(13.25-4399.75)	(66.00-1970.00)	(1763.00-1818.00)	(1307.00-1392.00)	(244.00-350.00)
OAT	24.50	113.00	95.00	173.00	135.00	465.00[a]	77.50[a]
	(17.75-26.25)	(26.50-439.00)	(43.50-427.50)	(61.25-918.50)	(52.50-1390.50)	(109.75-875.00)	(16.00-155.50)
O_3	25.50	564.00	474.00	209.00	259.00	139.00[a]	32.00[a]
	(12.00-37.25)	(75.00-1054.50)	(132.00-1025.50)	(126.50-1136.00)	(119.00-1377.00)	(57.50-538.00)	(25.00-70.00)
YC	18.00	1310.00[c]	662.00[c]	1610.50	1064.50	465.00[a]	77.50[a]
	(11.75-52.50)	(919.75-1740.25)	(355.00-2034.50)	(158.75-1682.50)	(235.25-2394.00)	(109.75-875.00)	(16.00-155.50)
O_3 + YC	26.50	28.50[f,h]	34.00[c,f,h]	33.50[a,d,f,h]	30.50[a,d,f,h]	23.50[a,d,f,g]	12.00[a,d,f,g]
	(20.00-32.75)	(23.50-41.75)	(27.25-40.25)	(27.75-37.75)	(26.50-38.75)	(20.00-36.00)	(3.75-12.50)
AST/ALT							
Control		0.78	1.36	5.71	0.06	0.05	0.10
		(0.45-1.38)	(0.28-6.94)	(0.13-11.29)	(0.03-0.09)	(0.03-0.07)	(0.09-0.10)
OAT		0.80	0.49	0.41	0.43	0.57	0.99[a]
		(0.50-2.97)	(0.17-0.85)	(0.18-0.70)	(0.10-0.77)	(0.15-0.79)	(0.29-1.70)
O_3		1.02	0.81	0.45	0.62	0.71[a]	1.52[a]
		(0.77-1.35)	(0.38-1.36)	(0.15-0.65)	(0.22-0.94)	(0.33-1.05)	(0.75-2.24)
YC		0.73	1.09	0.43	0.21	0.24	1.00[a]
		(0.48-1.11)	(0.62-7.86)	(0.26-0.78)	(0.07-0.45)	(0.07-0.87)	(0.20-2.02)
O_3 + YC		2.39[b,f,h]	2.08[d,f]	1.09[d,f,h]	1.39[a,d,f,h]	1.43[a,d,f,g]	2.08[a,c]
		(1.56-2.59)	(1.43-2.17)	(1.00-1.70)	(1.26-1.79)	(1.04-1.92)	(1.77-3.79)
TBIL/(μmol/L)							
Control	2.10	7.15	9.80	6.65	6.10	4.95	1.80
	(0.18-4.73)	(2.73-31.83)	(6.15-33.85)	(6.60-6.70)	(5.30-6.90)	(4.70-5.20)	(0.60-3.00)
OAT	3.80	5.20	5.30	6.55	3.50[a]	5.45	3.30
	(0.85-4.70)	(2.50-6.40)	(1.90-14.30)	(3.35-21.15)	(2.05-4.20)	(1.88-10.90)	(1.20-6.08)
O_3	1.55	4.30	4.30	3.30	1.90[a]	2.50[a]	3.00
	(0.20-4.85)	(2.45-5.65)	(3.05-7.05)	(1.95-6.45)	(1.10-4.35)	(1.85-4.20)	(0.80-4.95)
YC	2.65	3.40	7.00	3.40[a]	4.45	1.95[a]	2.70
	(0.35-5.18)	(1.60-26.15)	(2.95-33.65)	(1.15-3.55)	(1.55-5.78)	(0.80-3.93)	(0.98-10.73)
O_3 + YC	4.20	1.95[a,c,e]	1.00[b,c,e,h]	1.45[a,c]	1.30[a,d,g]	0.85[a,c,e]	3.50
	(3.83-4.33)	(0.10-2.40)	(0.30-2.38)	(0.80-5.10)	(0.70-1.55)	(0.50-2.13)	(0.50-4.28)
PT/s							
Control	6.80	13.20	18.90	12.25	8.70	7.80[a]	8.55[a]
	(6.50-13.05)	(8.93-38.93)	(12.40-60.70)	(11.70-12.80)	(8.40-9.00)	(7.60-8.00)	(8.20-8.90)
OAT	6.50	7.20[a]	7.85[a]	7.95	6.50	6.50	6.50
	(6.50-7.28)	(6.50-8.35)	(7.60-9.83)	(6.50-9.40)	(6.50-8.70)	(6.50-6.50)	(6.95-7.93)
O_3	7.30	8.60	9.50	7.80[a]	7.30	7.50	7.10[a]
	(6.95-8.03)	(7.30-11.35)	(8.65-16.35)	(7.50-10.00)	(6.90-8.45)	(7.00-7.70)	(6.80-7.30)
YC	7.70	12.55	9.90	7.40[a]	7.20	7.55	6.90
	(7.18-8.20)	(8.23-24.75)	(8.70-36.40)	(7.20-10.60)	(6.68-8.70)	(6.68-8.20)	(6.50-8.13)
O_3 + YC	7.65	7.25[a,c,g]	7.40[b,c,f,h]	7.30[a]	7.30[a]	6.85[a]	7.45[a]
	(7.35-8.15)	(6.50-8.13)	(7.20-8.65)	(7.18-8.28)	(7.28-7.40)	(6.50-7.50)	(7.03-7.70)
AMMO/(μmol/L)							
Control	69.55	96.50	114.80	132.80	143.40	53.00	91.40
	(35.47-106.35)	(53.20-161.33)	(80.90-133.90)	(82.30-183.30)	(67.40-219.40)	(32.50-73.50)	(40.60-142.20)
OAT	41.20	54.70	44.35[a]	49.85[a]	55.10[a]	49.65	33.30
	(35.45-54.50)	(43.70-79.70)	(25.93-83.10)	(38.48-62.28)	(31.35-64.53)	(38.55-75.45)	(13.40-52.50)
O_3	49.75	54.70	51.50[a]	65.70[a]	48.30	65.40	47.10
	(39.33-62.03)	(52.40-76.65)	(43.00-73.50)	(52.20-72.40)	(44.85-74.05)	(44.50-94.80)	(32.63-63.75)
YC	47.50	53.80	54.70[a]	55.70	77.35	45.40	45.50
	(34.25-73.23)	(49.45-173.23)	(43.70-79.70)	(45.83-86.95)	(57.63-168.25)	(39.18-97.68)	(23.35-110.85)
O_3 + YC	63.50	77.35	55.30[a]	59.65[a]	58.40	67.10	41.90
	(15.60-74.78)	(56.73-158.20)	(44.90-69.58)	(41.70-63.45)	(45.95-69.25)	(51.20-93.95)	(37.08-61.48)
BUN/(mmol/L)							
Control	3.30	5.50	3.80	2.40	3.60	3.65	5.15
	(2.45-4.45)	(3.85-7.15)	(2.70-6.20)	(1.60-3.20)	(3.10-4.10)	(3.20-4.10)	(1.20-9.10)
OAT	3.85	6.20	4.50	3.70	4.65	3.20	2.75
	(3.53-4.38)	(5.20-8.30)	(4.10-6.25)	(3.25-4.60)	(3.10-7.33)	(2.38-6.65)	(2.10-4.00)
O_3	3.75	5.70	5.80	4.00	3.90	2.60	2.60
	(3.18-5.55)	(4.65-8.70)	(4.75-7.05)	(3.20-5.80)	(2.40-6.45)	(1.90-6.35)	(2.20-3.30)
YC	3.50	7.55	4.30	4.10	3.00	2.00	3.20
	(2.88-5.73)	(3.90-13.58)	(3.55-13.00)	(1.98-7.13)	(2.55-6.68)	(1.68-6.75)	(2.83-3.20)
O_3 + YC	2.85	3.10[d,e,g]	3.50[e]	3.75	3.45	2.20	1.80[g]
	(2.28-3.65)	(1.50-3.98)	(2.70-4.15)	(2.30-4.68)	(3.30-3.95)	(1.80-3.28)	(1.70-2.40)

[a]$P < 0.05$ *vs* Control; [b]$P < 0.01$ *vs* Control; [c]$P < 0.05$ *vs* OAT; [d]$P < 0.01$ *vs* OAT; [e]$P < 0.05$ *vs* O_3; [f]$P < 0.01$ *vs* O_3; [g]$P < 0.05$ *vs* YC; [h]$P < 0.01$ *vs* YC. ALT of each dog was within the normal level (ALT < 60.0 U/L)[5] pre-injection, and thus the values of AST/ALT made no sense at that time.





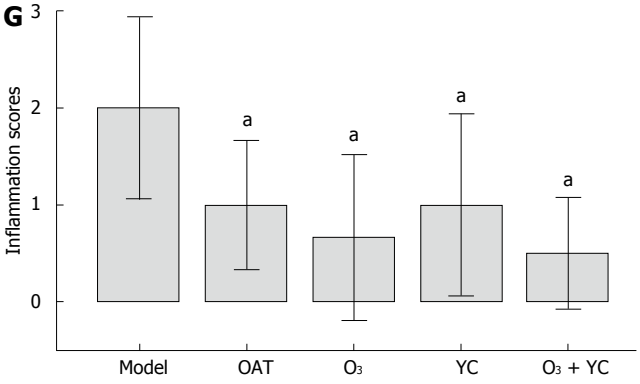


Figure 2 Histological assessment of livers following death or living for 15 d (original magnification, × 100, **A**, **B**; × 400, **C**, **D**, **E**). Hepatocellular bridged necrosis, mononuclear and lymphoid inflammatory infiltration, necrosis and hemorrhage (**A**) accompanied reticular fibers structures collapsing in control group (**B**). Increased mononuclear and lymphoid inflammatory infiltration, and necrosis in groups OAT, O_3, and YC (**C**, **D**) compared with group O_3 + YC (**E**). Inflammation and necrosis were graded on a 4-point scale (Table 1) by a blinded pathologist in 20 random high power fields per animal (n = 6 in each group), represented graphically in panels (**F**) errorbars: 95.00% CI and (**G**) errorbars: 95.00% CI. Data represent mean ± SE; $^aP < 0.05$ *vs* Control.

medical ozone to treat infectious diseases, including viral hepatitis B, and to treat rheumatic diseases. Although effort has been done to elucidate the mechanism of ozonetherapy, but its practices in whole still remained in an empirical fashion and have not been accepted by sutra legitimacy medicine.

Research frontiers

Drug-induced liver injury is an increased important problem in modern medical practice. Apart from avoiding using hepatoxic drugs, none of effective methods to prevent drug induced hepatitis are confirmed so far. There are several articles that introduced using medical ozone to prevent liver or renal ischaemia and reperfusion (I/R) injury in animal models. Free radical and reactive oxygen species during I/R process are the main cause of organ I/R injury. The mechanism of some drug-induced hepatitis is the same way as I/R injury. So, the ozone's property of inducing antioxide enzymes and enhancing capacity of radical scavenger should confer preventive effect for protecting liver from hepatoxic drug-induced injury.

Innovation and breakthroughs

This study gives us a new knowledge of medical ozone and impels us to have following deduction, i.e. medical ozone and herb medicine rectal administration may be a new strategy to prevent drug-induced hepatitis. From design point of view, big animal as dog as animal model in this study imitates human being more likely than small canine as mouse. Rectal administration of ozone and herb medicine was proved effective in this experiment, and provided convenient way for future clinical application.

Application

The results from this animal study confirm the protective effect of ozone and prescription of traditional Chinese medicine on hepatoxic drug-induced liver injury. It encourages us to apply medical ozone in preventing some drug-induced hepatitis in the future clinical trials.

Termiology

Medical ozone is a mixture gas of ozone and oxygen. It is made from pure oxygen by an ozone generator. This machine used in medical therapy must have capacity to adjust ozone concentration in a precision and stabilization pattern.

Peer review

This study further confirmed the protective effect of medical ozone and herb medicine on drug-induced hepatitis and was a steady step toward its clinical application. Medical ozone and traditional Chinese medicine are both ancient antiques, its worthiness was not staying in ancient history and its merits are also useful in modern medicine, just simply needs to be added modern decoration, i.e. the evidence based medicine (EBM).

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CLINICAL RESEARCH

Selective decrease in colonic CD56$^+$ T and CD161$^+$ T cells in the inflamed mucosa of patients with ulcerative colitis

Masaru Shimamoto, Yoshitaka Ueno, Shinji Tanaka, Toshiko Onitake, Rie Hanaoka, Kyoko Yoshioka, Tsuyoshi Hatakeyama, Kazuaki Chayama

Masaru Shimamoto, Toshiko Onitake, Rie Hanaoka, Kyoko Yoshioka, Tsuyoshi Hatakeyama, Kazuaki Chayama, Department of Medicine and Molecular Science, Hiroshima University, Hiroshima 734-8551, Japan
Yoshitaka Ueno, Shinji Tanaka, Department of Endoscopy, Hiroshima University Hospital, Hiroshima Japan
Correspondence to: Yoshitaka Ueno, MD, PhD, Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. yueno@hiroshima-u.ac.jp
Telephone: +81-82-2575193 Fax: +81-82-2575194
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Abstract

AIM: To investigate the role of local colonic mucosal NK receptor-positive T (NKR$^+$ T) cells in the regulation of intestinal inflammation, we analyzed the population and function of these cells in ulcerative colitis (UC).

METHODS: Colonic mucosal tissues were obtained from colonoscopic biopsies of the descending colon from 96 patients with UC (51 endoscopically uninflamed, 45 inflamed) and 18 normal controls. Endoscopic appearance and histologic score at the biopsied site were determined by Matts' classification. A single cell suspension was prepared from each biopsy by collagenase digestion. Two NKR$^+$ T cell subsets, CD56$^+$ (CD56$^+$CD3$^+$) T cells and CD161$^+$ (CD161$^+$CD3$^+$) T cells, were detected by flow cytometric analysis. Intracellular cytokine analysis for anti-inflammatory cytokine interleukin-10 (IL-10) was performed by *in vitro* stimulation with phorbol-myristate-acetate (PMA) and ionomycin.

RESULTS: CD56$^+$ T cells and CD161$^+$ T cells are present in the normal human colon and account for 6.7% and 21.3% of all mononuclear cells, respectively. The populations of both CD56$^+$ T cells and CD161$^+$ T cells were decreased significantly in the inflamed mucosa of UC. In contrast, the frequency of conventional T cells (CD56$^-$CD3$^+$ cells and CD161$^-$CD3$^+$ cells) was similar among the patient and control groups. The populations of NKR$^+$ T cells were correlated inversely with the severity of inflammation, which was classified according to the endoscopic and histologic Matts' criteria. Interestingly, approximately 4% of mucosal NKR$^+$ T cells expressing IL-10 were detected by *in vitro* stimulation with PMA and ionomycin.

CONCLUSION: Selective reduction in the population of colonic mucosal NKR$^+$T cells may contribute to the development of intestinal inflammation in UC.

Key words: Natural killer T cells; Ulcerative colitis; Interleukin-10

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INTRODUCTION

Human ulcerative colitis (UC) is a chronic relapsing disorder of ill-defined immunoregulatory dysfunction that leads to inflammation or ulceration of the intestinal tract[1]. Increasing evidence suggests that dysregulation of mucosal T cells may play a key role in the pathogenesis of UC, which results in secretion of proinflammatory mediators, accumulation of inflammatory cells, and tissue damage[2-4]. The pathogenesis of UC remains obscure, but it is now believed that dysfunction of immunoregulatory T cells is considered one of the mechanisms by which intestinal inflammation persists in UC[5].

Human T cells that express natural killer (NK) markers, including CD56 and CD161, were originally discovered in the liver. They differentiate extrathymically and are considered immunoregulatory T cells[6,7]. Hepatic CD56$^+$ T cells and CD161$^+$ T cells can rapidly produce Th1-type and Th2-type cytokines, suggesting that these cells have roles in the regulation of both innate and adoptive immune responses[8-10]. They also have cytotoxic activity against some cancer cell lines[11]. Several investigators reported that hepatic CD56$^+$ T cells and CD161$^+$ T cells are depleted significantly in liver with chronic hepatitis C virus infection[12,13], suggesting that these cells may be involved in the development of hepatic inflammation.

These NK receptor-positive (NKR$^+$) T cells include both conventional major-histocompatibility complex (MHC)-restricted T cells that recognize peptide antigens[14]

and so-called NKT cells, which recognize glycolipid antigens presented by a non-classical antigen-presenting molecule, CD1[15-17]. The latter cell type frequently expresses invariant T cell receptor α-chains (Vα24JαQ in humans and Vα14Jα18 in mice)[9,18].

Recently it was reported that these NKR^+ T cells reside in the human intestine[19], and these populations are reduced in the colonic mucosa of patients with colorectal cancer[20]. However, to date, there have been no reports of the roles of these cells in modulation of intestinal inflammation in UC. Here, we investigated the relation between these cell populations and the severity of colonic inflammation in patients with UC by assessing colonic mononuclear cells in biopsy specimens. We found that a selective decrease in the population of colonic NKR^+ T cells may be involved in the progression of local colonic mucosal inflammation in UC.

MATERIALS AND METHODS

Study groups

Demographic features of the patients and history of drug therapy up to the time of colonoscopy are summarized in Table 1. The diagnosis of UC was based on established endoscopic and histopathologic criteria[21]. Colonic mucosal tissues were obtained from colonoscopic biopsies of 96 patients with UC (45 inflamed, 51 uninflamed). The control group consisted of 18 patients. Ten of these control patients presented with a chief complaint of abdominal pain in which a histological diagnosis was not established. The remaining eight control patients were evaluated endoscopically for hematochezia and found to have solitary adenomatous polyps. All control colonic mucosal samples were taken from a histologically normal portion of the biopsied specimen at least 10 cm away from the involved sites with the polyps. The inflammatory activity of these areas was evaluated endoscopically and histologically according to Matts' criteria with some modifications[22,23]. Inflamed and uninflamed areas were defined as grades 2-4 (and 5 for histologic Matts' criteria) and grade 1, respectively. All biopsy specimens were obtained from the descending colon. Standard 2.8 mm biopsy forceps (Olympus Optical, Tokyo, Japan) were used through all colonoscopes. All samples were obtained with informed consent in accordance with the Helsinki Declaration.

Table 1 Demographic features of participating patients with UC

		Ulcerative colitis	
	Normal	Inflamed	Uninflamed
No. of patients (cases)	18	45	51
Sex (male/female)	11/7	31/14	35/16
Age (yr), mean ± SD	47.5 ± 20.1	35.3 ± 13.6	39.3 ± 14.1
Disease duration (yr)		6.5 ± 6.6	6.2 ± 5.5
Types of colitis			
Total	-	31	10
Left-sided	-	9	14
Proctitis	-	0	24
Others	-	5	3
Treatment			
Prednisolone (PSL) (no/yes)	-	15/30	29/22
Azathioprine (AZA) (no/yes)	-	31/14	42/9

Isolation of lamina propria mononuclear cells

All specimens were weighed prior to isolation of colonic mononuclear cells (MNCs). Five biopsy samples for purification of MNCs and two biopsy samples for evaluation of histology were taken from the same region of each colon, and MNCs were purified as described previously[24]. Briefly, specimens were digested with 150 U/mL of collagenase in RPMI medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS, Sigma), 100 mg/L of gentamicin (Gibco, Gaithersburg, MD), 500 μg/L of penicillin, and 500 μg/L of streptomycin (Gibco) at 37℃ for 90 min. The cells were pelleted and washed with cold phosphate-buffered saline (PBS). Cells were resuspended in 44% isotonic Percoll (Sigma) underlaid with 66% isotonic Percoll and centrifuged for 20 min at 2200 r/min at room temperature. Cells at the interface were collected and washed twice with cold PBS. Cell viability was determined by 0.1% trypan blue dye exclusion, and it was consistently > 90% in all of the patient groups.

Intestinal histology

Intestinal biopsy specimens were fixed immediately in 10% formalin in sodium phosphate buffer and sent to the Department of Pathology at Hiroshima University for processing. Biopsies were embedded in paraffin, and histological sections were stained with hematoxylin and eosin for evaluation. Inflammation was graded according to Matts' classification[22]. The pathologist was blinded to cell surface analysis data.

Flow cytometric analysis

Cells were incubated with a saturating amount of FITC-conjugated anti-human CD3 (UCHT1) mAb and phycoerythrin (PE)-conjugated anti-CD56 (B159) mAb or anti-CD161 (DX12) mAb. All staining reagents were obtained from Becton Dickinson (BD, San Jose, CA, USA). After cells were washed twice with PBS, the stained cells were analyzed on a FACScan (BD), and data were processed with Cell Quest software (BD). The relative proportions of the lymphocyte subpopulations were determined as percentages of the total numbers of cells in a lymphogate defined by forward and side scatter properties. Non-viable cells were excluded by detection of propidium iodide uptake.

Stimulation of cells and staining for intracellular cytokines

Freshly isolated MNCs isolated from a patient with active UC were suspended in complete RPMI medium at a density of 1×10^6 cells/mL and stimulated for 6 h in 96-well plates (Microtest™ 96, BD) at 37℃ in 5% CO_2. Cells were stimulated with 50 ng/mL phorbol-myristate-acetate (PMA) plus 500 ng/mL ionomycin. As controls, unstimulated cells were treated similarly. Interleukin-10 (IL-10) production by NKT cells was examined by a combination of cell-surface and intracytoplasmic mAb staining for IL-10 (JES3-19F1, BD) with Cytofix/Cytoperm Plus™ (BD) and analyzed by flow cytometry.

Statistical analysis

Data were analyzed with StatView software (Japanese version, Hulinks, Tokyo, Japan) on a Macintosh Computer

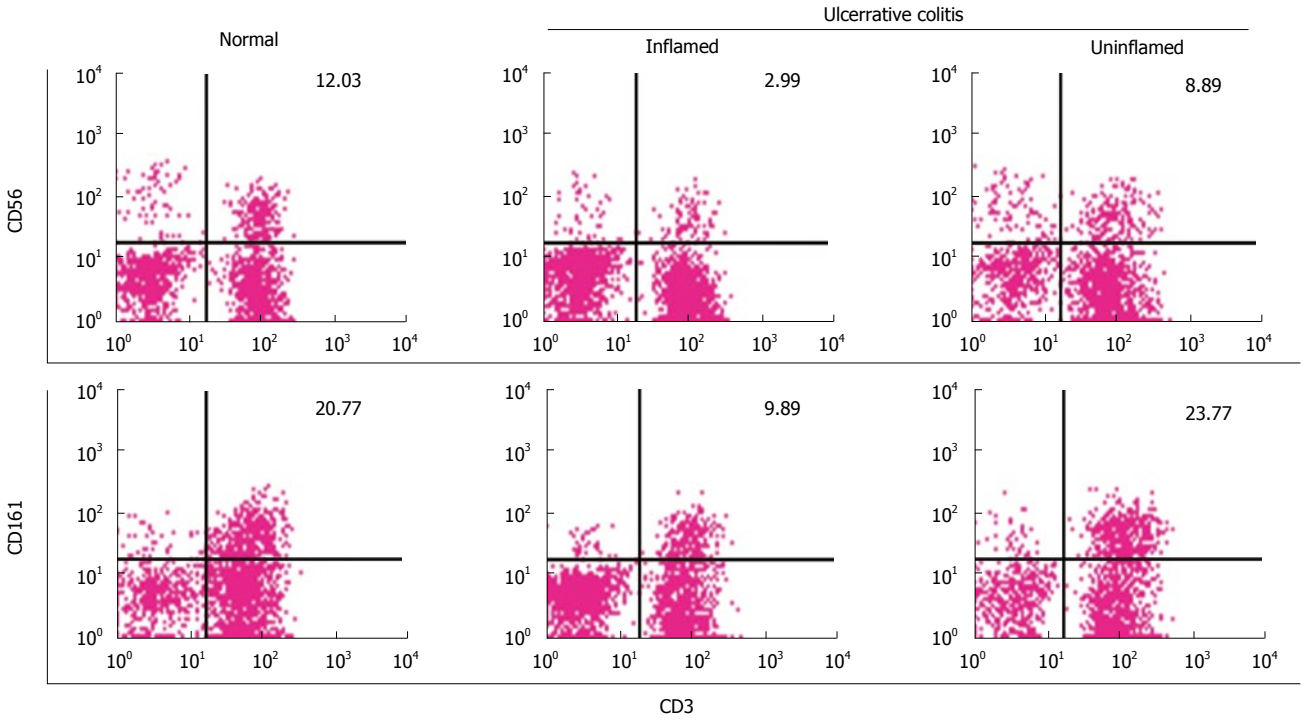

Figure 1 Representative FACS profiles of human colonic mucosal NKT cells. Flow cytometric analysis of CD3 and CD56/CD161 expression on MNCs isolated from colonic samples showing normal mucosa (left), inflamed (Matts' grade 3b) UC mucosa (middle), and uninflamed (Matts' grade 1) UC mucosa (right). The numbers in the top right quadrants denote the percentages of $CD56^+$ T and $CD161^+$ T cells.

(Apple Computer, Cupertino, CA). Data are expressed as mean ± SD. Differences between groups were examined for statistical significance with Student *t* test after analysis of variances (ANOVA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Decreases in $CD56^+$ T cells and $CD161^+$ T cells in the inflamed colonic mucosa of patients with UC

Representative FACS patterns of NKR^+T cells are shown in Figure 1. Flow cytometric analysis of freshly isolated colonic LPLs revealed that the proportion of NKR^+T cells expressing CD56 was significantly lower in patients with active UC (3.3% ± 1.7%, $P < 0.0001$; Figure 2A) in comparison with controls (6.7% ± 3.2%) and patients with inactive UC (7.5% ± 3.9%). Similarly, the proportion of NKR^+T cells expressing CD161 was significantly lower in patients with active UC (13.4% ± 6.4%, $P < 0.0005$; Figure 2B) in comparison with controls (21.3% ± 7.7%) and patients with inactive UC (21.4% ± 1.5%). The proportion of $CD3^-CD56^+$ NK cells was also reduced significantly in colonic LPL from patients with active UC in comparison with controls and patients with inactive UC (data not shown). No significant differences in the proportions of $CD56^-CD3^+$ and $CD161^-CD3^+$ conventional T cells were observed among these groups (Figure 2C and D), suggesting that mucosal NKR^+ T cells were selectively decreased in the inflamed UC mucosa. Furthermore, when we considered the inflamed mucosa by endoscopic and histologic classifications of Matts' grade, the populations of these NKR^+ T cells decreased as the degree of inflammation increased (Figures 3 and 4A). In contrast, the proportions of $CD56^-CD3^+$ and $CD161^-CD3^+$ conventional T cells were not influenced by the degree of histologic intestinal inflammation (Figure 4B). These results suggest that a relative decrease in the population of colonic NKR^+ T cells may exacerbate intestinal inflammation.

Prednisolone and azathioprine therapies did not affect the percentage of colonic NKR^+ T cells

The percentages of colonic NKR^+ T cells were compared between UC patients treated with or without prednisolone (PSL). We separated the groups into patients with inflamed and uninflamed mucosa because inflammation affects the proportion of NKR^+ T cells. PSL treatment did not influence the proportion of colonic NKR^+ T cells (Figure 5A and B). PSL dose did not influence the population of NKR^+ T cells (data not shown). Treatment with azathioprine (AZA) also did not affect the proportion of NKR^+ T cells (Figure 5C and D).

In vitro stimulation with PMA and ionomycin results in IL-10 production by colonic NKR^+ T cells

To further explore the functional role of NKR^+ T cells, analysis of expression of the anti-inflammatory cytokine, IL-10 was performed. As shown in Figure 6, a subset of colonic NKR^+ T cells from normal colonic mucosa produced intracellular IL-10 when stimulated *in vitro* with PMA^+ ionomycin.

DISCUSSION

In the present study, we observed that the populations of $CD56^+$ T cells and $CD161^+$ T cells were decreased significantly in inflamed lesions on the colonic mucosa

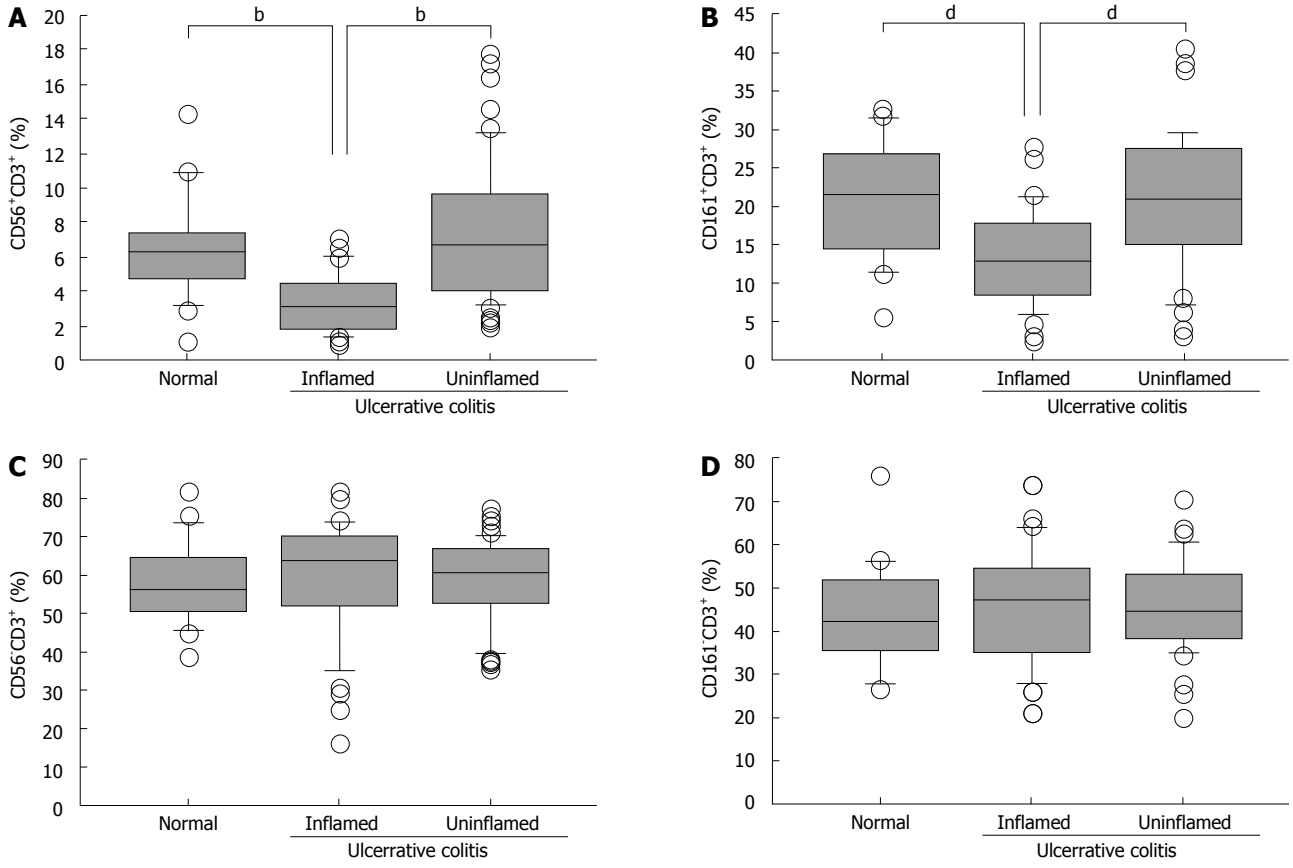

Figure 2 Selective decreases in the proportions of $CD56^+CD3^+$ (**A**) and $CD161^+CD3^+$ (**B**) cells in inflamed colonic mucosa of patients with UC. Box plot graphical representation of the percentage of colonic NKR^+ T cells from normal mucosa of non-UC patients (n = 18), uninflamed mucosa (endoscopic Matts' grade 1, n = 51), and inflamed mucosa (Matts' grades 2-4, n = 45). $^bP < 0.0001$, $^dP < 0.0005$; inflamed mucosa *vs* normal or uninflamed mucosa. (**C, D**) No relation was observed between the percentage of conventional T cells ($CD56^-CD3^+$ or $CD161^-CD3^+$ cells) and the degree of endoscopic inflammation.

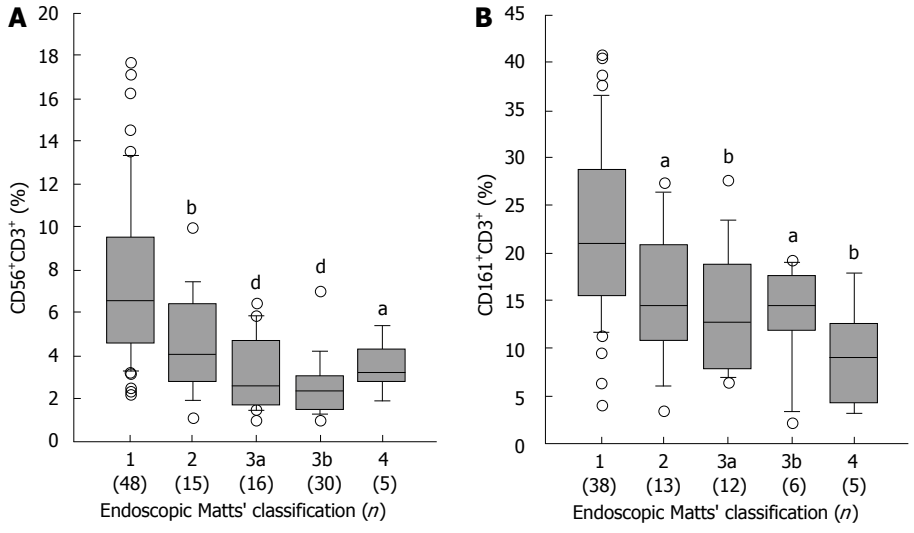

Figure 3 Relation between Matts' scores for endoscopy and the percentage of NKR^+ T cells. Box plot graphical representation of the percentage of colonic NKR^+ T cells in UC patients. The percentage of colonic NKR^+ T cells was compared with the Matts' scores. "n" indicates the number of cases. $^aP < 0.05$, $^bP < 0.01$, $^dP < 0.0001$, *vs* Matts' 1.

in patients with UC. The proportions of these cells were inversely well correlated with the degree of endoscopic and histologic intestinal inflammation. In contrast, the percentages of conventional T cells were similar among our study groups. Our findings suggest that selective decreases in levels of colonic NKR^+ T cells may contribute to the progression of intestinal inflammation.

$CD56^+$ T cells and $CD161^+$ T cells were originally identified in human liver. These cells have an extrathymic origin and have properties of innate lymphocytes[6,7]. These cells are uniquely capable of rapidly producing Th1 and Th2 cytokines upon stimulation[8-10], indicating a broad role for these cells in the activation and regulation of multiple arms of the immune responses. Although the functions of these $CD56^+$ T cells and $CD161^+$ T cells are currently unknown, they express memory T cell phenotypes[15] and homing chemokine receptors[25], suggesting that they may be memory T cells. The proliferation and function of these

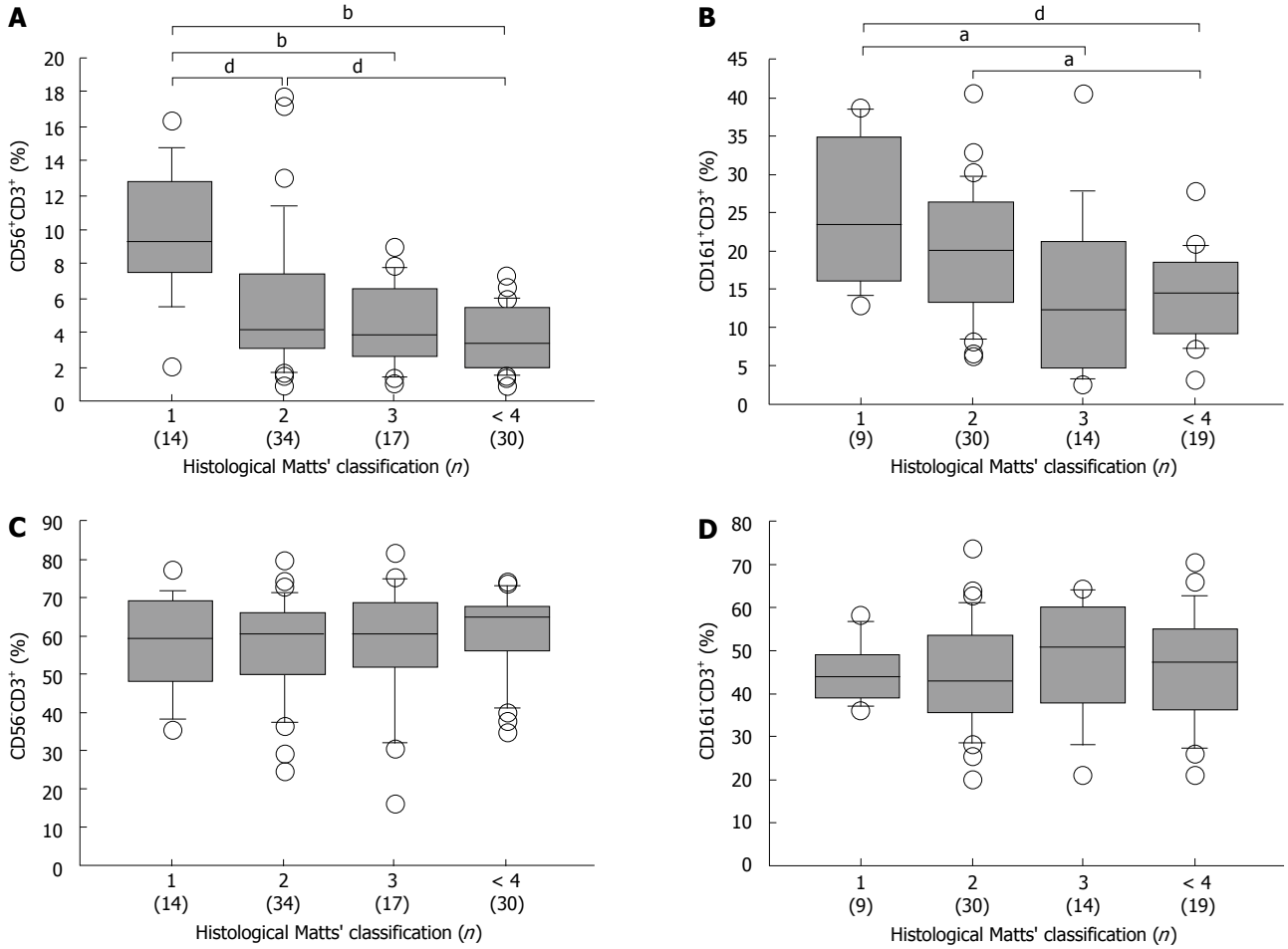

Figure 4 Relation between the histologic Matts' scores and the percentage of NKR$^+$ T cells. Biopsy samples for evaluation of histology were taken from the same regions as samples for MNC purification were taken. The percentage of colonic NKR$^+$ T cells was compared with the histologic Matts' score. Levels of CD56$^+$ T cells (**A**) and CD161$^+$ T cells (**B**) in the colon decreased significantly as a function of the severity of inflammation. "*n*" indicates the number of cases. $^b P < 0.0001$, $^d P < 0.005$ *vs* Matts' 1, $^a P < 0.05$ *vs* Matts' 1 or Matts' 2. No relation was detected between the percentages of conventional T cells (CD56$^-$CD3$^+$ or CD161$^-$CD3$^+$ cells) in the colon and the severity of histological inflammation (**C**, **D**).

cells are regulated by various cytokines, including IL-15 and granulocyte-macrophage colony-stimulating factor[26,27]. These NKR$^+$ T cells include invariant NKT cells that recognize glycolipid antigens presented by the non-classical antigen-presenting molecule CD1[15-17]. CD1d, which is an MHC Class I-like molecule, is expressed by human intestinal epithelial cells[28,29]. Bendelac *et al* reported that Vα24 NKT cells recognize CD1d molecules and act as immunoregulatory cells by producing various cytokines[30]. Blumberg *et al* showed that the CD1d molecule expressed by intestinal epithelial cells is functional because ligation with antibody against CD1d induces production of IL-10 by an intestinal epithelial cell line, T84[31]. Therefore, the interaction between CD1d on intestinal epithelial cells and mucosal CD1d-restricted T cells may be important for maintaining intestinal homeostasis. Whether these NKR$^+$ T cells are CD1d-restricted is currently under investigation.

The role of NKR$^+$ T cells in the development of intestinal inflammation is not clear. A mouse study revealed that CD1d-α-galactosylceramide (αGalCer)-restricted NKT cells are critical for protection against the development of dextran sulfate sodium-induced colitis[32]. The findings of another study suggested that IL-13-producing NKT cells are involved in the development of oxazolone-induced colitis in mice[33]. These data suggest that NKT cells are crucial for eliciting protective immunity against intestinal inflammation.

Although NKT cells are known to have an anti-inflammatory role[34], what is the mechanism by which these cells regulate the immune system? Sonoda *et al* showed that about 5% of NKT cells have the capacity to produce the immunosuppressive cytokine IL-10[35]. We have shown here that some colonic mucosal NKR$^+$ T cells can produce IL-10 when stimulated *in vitro*. IL-10 expression is also important for mucosal immunologic homeostasis. IL-10-deficient mice show enhanced production of colonic proinflammatory cytokines, including IFN-γ, and develop spontaneous chronic enterocolitis[36,37]. The subset of regulatory T cells that produce IL-10 suppresses the development of experimental intestinal inflammation[38]. Furthermore, mice with a macrophage/neutrophil-specific disruption of the Stat3 gene show impaired IL-10-mediated functions and develop chronic enterocolitis[39]. These observations support the idea that mucosal immune homeostasis involves localized production of molecules that promote IL-10 expression by resident immunoregulatory cells in the mucosa. Therefore, decreased IL-10 production due

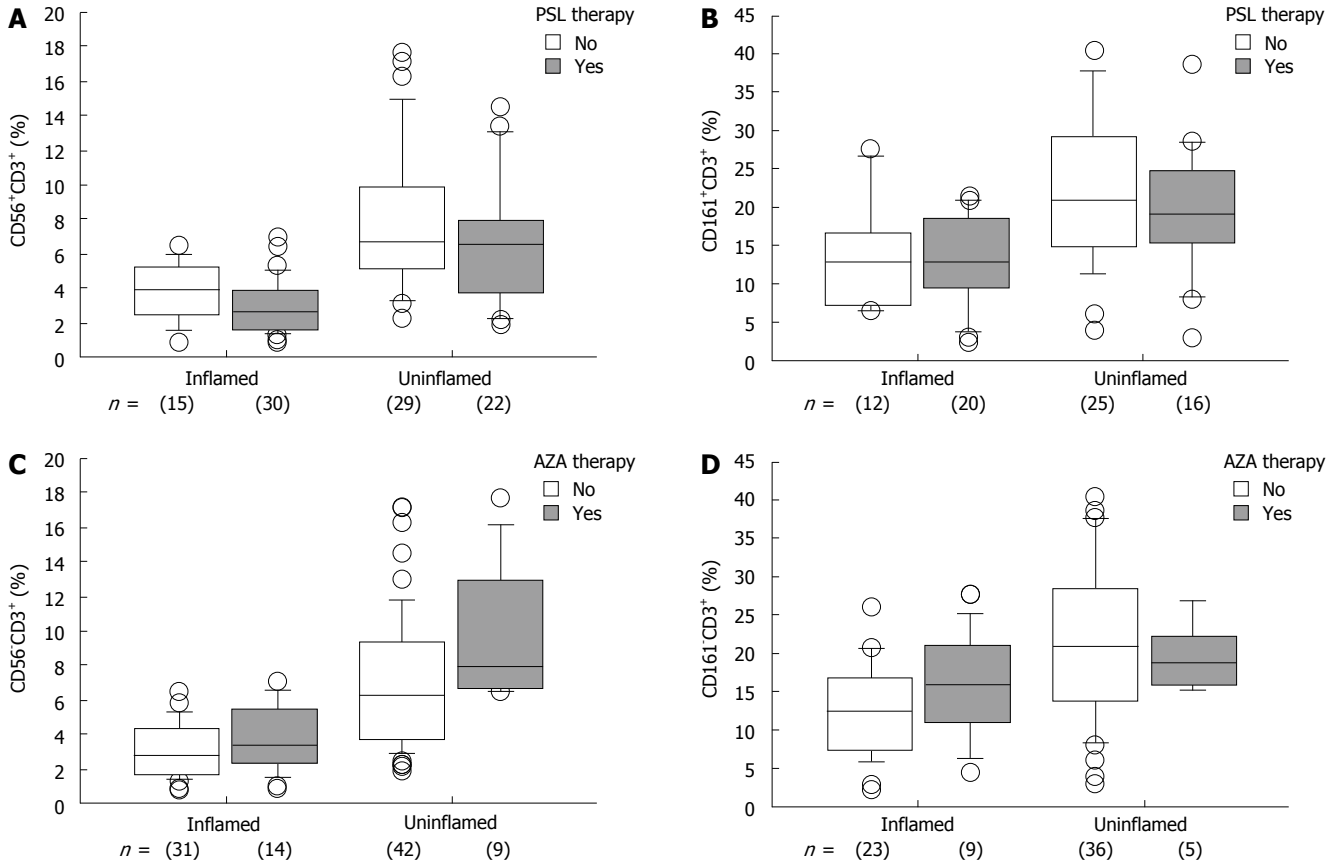

Figure 5 The percentage of colonic NKR$^+$ T cells was not affected by treatment with PSL or AZA. The percentages of colonic NKR$^+$ T cells were compared between untreated UC patients and those treated with PSL (**A, B**). The level of NKR$^+$ T cells was compared between untreated UC patients and those treated with AZA (**C, D**). "*n*" indicates the number of cases.

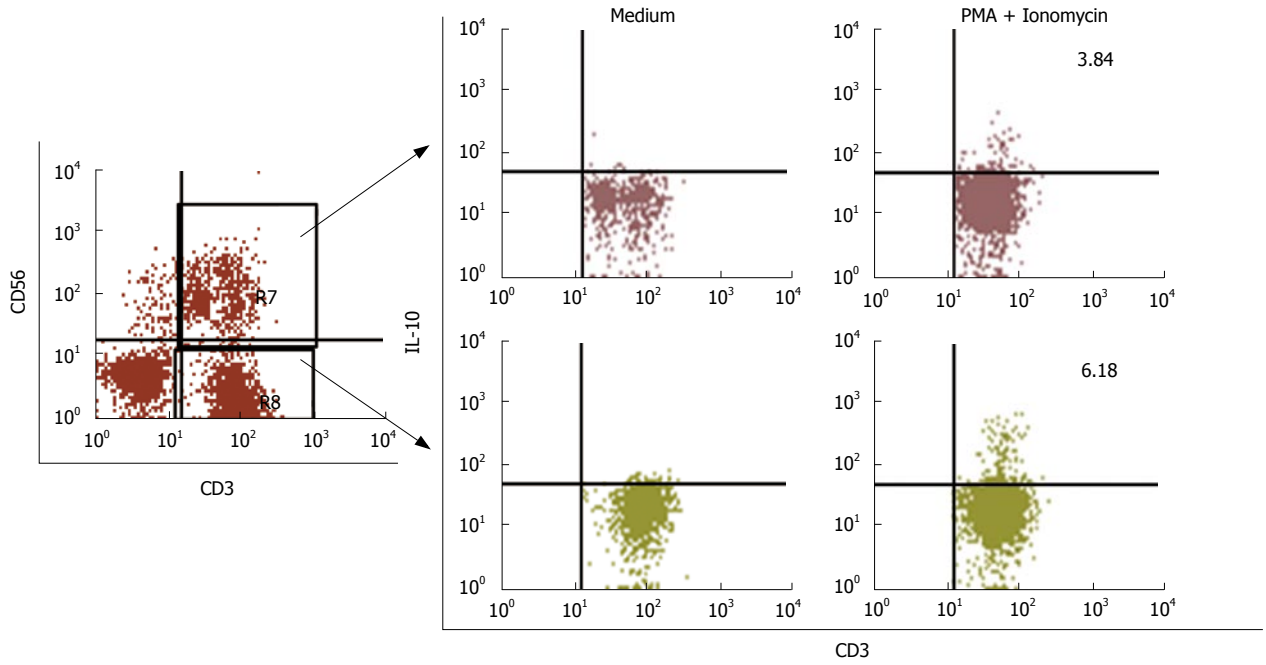

Figure 6 Detection of IL-10-producing NKR$^+$ T cells in the colonic mucosa. Flow cytometric analysis of IL-10 production by *ex vivo*-stimulated colonic MNCs isolated from a patient with active UC. FACScan was gated on CD56$^+$CD3$^+$ cells. The numbers indicate the percentages of CD56$^+$CD3$^+$ cells producing cytokines relative to unstimulated control cells.

to depletion of NKR$^+$ T cells in the colon of human UC as observed in the present study may result in insufficient inhibition of pathologic T cells and activated macrophages. Further studies are needed to compare the percentage of IL-10-producing cells between normal and UC colons.

Several mechanisms may account for the decreased

proportions of NKR^+ T cells in UC. First, the observed depletion of the local colonic NKT cell population may be the result of a continuous process of activation-induced cell death[40]. Second possible mechanism may be the loss of surface NK markers. It was recently reported that NKT cells activated by glycolipid antigens down-regulate NK receptors[41]. Third mechanism may be impaired recruitment of NKR^+ T cells from the peripheral circulation. $CD56^+$ T cells express chemokine receptors such as CCR5 or homing receptors such as $\alpha4\beta7$, a ligand for MAdCAM1 expressed specifically on the intestinal high endothelial venules[25]. These issues are currently under investigation in our laboratory.

In conclusion, human colonic $CD56^+$ T cells and $CD161^+$ T cells are thought to play important roles as anti-inflammatory cells, and the decrease in the proportions of these cells in inflamed lesions of the colon may be one mechanism by which colonic inflammation progresses in UC.

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COMMENTS

Background

Human T cells that have natural killer markers are mainly located in the liver and considered immunoregulatory T cells. Recently it was reported that these natural killer receptor (NKR^+) T cells reside in the human intestine. But the exact significance of these cells in the intestine is unknown. This study was aimed at investigating the changes and significance of these cells in human ulcerative colitis (UC).

Research frontiers

Previous studies have demonstrated the significance of NKR^+T cells in human colorectal cancer. However, the role of these cells in the chronic intestinal inflammation is undefined.

Innovations and breakthroughs

The populations of both $CD56^+$ T cells and $CD161^+$ T cells were decreased significantly in the inflamed mucosa of UC. The populations of these NKR^+ T cells were correlated inversely with the severity of inflammation, which was classified according to the endoscopic and histologic Matts' criteria. In contrast, the frequency of conventional T cells ($CD56^-CD3^+$ cells and $CD161^-CD3^+$ cells) was similar among the patients with UC and healthy groups.

Applications

Selective reduction in the population of colonic mucosal NKR^+ T cells may contribute to the development of intestinal inflammation in UC.

Terminology

CD56 and CD161 are known as natural killer cell surface antigens. CD56 was demonstrated to be a neural cell adhesion molecule-1 (NCAM-1). $CD56^+$T cells are believed to be major players in immunosurveillance and antitumor responses. CD161 is a human NKR-P1 family and is recognized as an analogue of murine NKR-P1C. Moreover, $CD161^+$ T cells are also known to play an important role for antitumor immunity. CD161 is also expressed by invariant natural killer T cells that are restricted to CD1d molecule on antigen presenting cells.

Peer review

In this study, the authors demonstrate that the proportion of NKR^+ T cells is selectively decreased in the colonic mucosa of UC patients and that this reduced cell number correlates well with the severity of the disease. These cells are capable of producing an anti-inflammatory cytokine, IL-10. This work adds important information regarding cellular subsets that might be involved in the pathogenesis of UC.

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CLINICAL RESEARCH

*NAT2*6A*, a haplotype of the *N*-acetyltransferase 2 gene, is an important biomarker for risk of anti-tuberculosis drug-induced hepatotoxicity in Japanese patients with tuberculosis

Norihide Higuchi, Naoko Tahara, Katsunori Yanagihara, Kiyoyasu Fukushima, Naofumi Suyama, Yuichi Inoue, Yoshitsugu Miyazaki, Tsutomu Kobayashi, Koh-ichiro Yoshiura, Norio Niikawa, Chun-Yang Wen, Hajime Isomoto, Saburou Shikuwa, Katsuhisa Omagari, Yohei Mizuta, Shigeru Kohno, Kazuhiro Tsukamoto

Norihide Higuchi, Naoko Tahara, Tsutomu Kobayashi, Kazuhiro Tsukamoto, Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan
Katsunori Yanagihara, Yoshitsugu Miyazaki, Hajime Isomoto, Saburou Shikuwa, Katsuhisa Omagari, Yohei Mizuta, Shigeru Kohno, Second Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki 852-8501, Japan
Kiyoyasu Fukushima, Division of Internal Medicine, Japanese Red Cross Nagasaki Genbaku Isahaya Hospital, Isahaya 859-0497, Japan
Naofumi Suyama, Department of Internal Medicine, Nagasaki Municipal Medical Center, Nagasaki 852-8012, Japan
Yuichi Inoue, Department of Internal Medicine, Isahaya Health Insurance General Hospital, Isahaya 854-8501, Japan
Koh-ichiro Yoshiura, Norio Niikawa, Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-12-4, Nagasaki 852-8523, Japan
Koh-ichiro Yoshiura, Norio Niikawa, Kazuhiro Tsukamoto, SORST, JST, Kawaguchi, Japan
Chun-Yang Wen, Department of Digestive Disease Center, Beihua University, Jilin 132013, Jilin Province, China
Supported by Grant-in-Aid for Scientific Research (Category B, No. 18390168) for K Tsukamoto by the Ministry of Education, Culture, Sports, Science and Technology of Japan
Correspondence to: Professor Kazuhiro Tsukamoto, Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan. ktsuka@nagasaki-u.ac.jp
Telephone: +81-95-8192447 Fax: +81-95-8192895
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Abstract

AIM: To investigate an association between *N*-acetyltransferase 2 (*NAT2*)-haplotypes/diplotypes and adverse effects in Japanese pulmonary tuberculosis patients.

METHODS: We studied 100 patients with pulmonary TB treated with anti-TB drugs including INH. The frequencies and distributions of single nucleotide polymorphisms, haplotypes, and diplotypes of *NAT2* were determined by the PCR-restriction fragment length polymorphism method, and the results were compared between TB patients with and without adverse effect, using multivariate logistic regression analysis.

RESULTS: Statistical analysis revealed that the frequency of a variant haplotype, *NAT2*6A*, was significantly increased in TB patients with hepatotoxicity, compared with those without hepatotoxicity [P = 0.001, odds ratio (OR) = 3.535]. By contrast, the frequency of a wild-type (major) haplotype, "*NAT2*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity (P < 0.001, OR = 0.265). There was no association between *NAT2*-haplotypes and skin rash or eosinophilia.

CONCLUSION: The present study shows that *NAT2* is one of the determinants of anti-TB drug-induced hepatotoxicity. Moreover, the haplotypes, *NAT2*4* and *NAT2*6A*, are useful new biomarkers for predicting anti-TB drug-induced hepatotoxicity.

Key words: Tuberculosis; Anti-tuberculosis drugs; Drug-induced hepatotoxicity; *NAT2*-haplotype; DNA-based diagnosis

Higuchi N, Tahara N, Yanagihara K, Fukushima K, Suyama N, Inoue Y, Miyazaki Y, Kobayashi T, Yoshiura K, Niikawa N, Wen CY, Isomoto H, Shikuwa S, Omagari K, Mizuta Y, Kohno S, Tsukamoto K. *NAT2*6A*, a haplotype of the *N*-acetyltransferase 2 gene, is an important biomarker for risk of anti-tuberculosis drug-induced hepatotoxicity in Japanese patients with tuberculosis. *World J Gastroenterol* 2007; 13(45): 6003-6008

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INTRODUCTION

Tuberculosis (TB) is a re-emerging infectious disease that was declared a global health problem by the World Health Organization in 1993[1]. Since there were 9 million new TB cases and approximately 2 million TB deaths in 2004, and more than 80% of all TB patients live in sub-Saharan Africa and Asia, the epidemiology and control of TB remain important public health issues[1,2]. However, the management of TB is associated with serious problems, including disease relapse in elderly patients, occurrence

in acquired immunodeficiency syndrome, development of adverse effects of anti-TB drugs, and increase in the prevalence of multidrug-resistant *Mycobacterium tuberculosis*[2-5]. In particular, poor compliance or non-compliance with anti-TB drugs because of adverse effects, such as hepatotoxicity, skin rash, drug fever, peripheral neuritis, eosinophilia, and/or hyperuricemia, may lead to decrease in the quality-of-life of TB patients and appearance of multidrug-resistant *M. tuberculosis*. An important focus of previous studies was drug-induced hepatotoxicity, because it constitutes a major and severe adverse effect in the treatment of tuberculosis. Although the common risk factors for hepatotoxicity include advanced age[6,7], gender[7-10], malnutrition[6,9], complications of diseases[8,10,11], and alcohol intake[6,8,12], genetic factors also have an important impact on the likelihood of the development of drug-induced hepatotoxicity. Case-control studies with candidate genes in the affected populations have identified several possible susceptibility genes, e.g., *N*-acetyltransferase 2 (*NAT2*)[13-17], cytochrome P450 2E1 (*CYP2E1*)[16,18], glutathione *S*-transferase M1 (*GSTM1*)[16,19], glutathione *S*-transferase T1 (*GSTT1*)[16,19], and HLA-DQA1/-DQB1[20].

We focused our research on *NAT2* as a candidate gene associated with drug-induced hepatotoxicity because *NAT2* is the main enzyme involved in isoniazid (INH) metabolism, and is expressed in the liver. Diminution or disturbance of *NAT2* activity could result in the accumulation of precursors, such as hydrazine and acetylhydrazine in the liver, leading to hepatotoxicity[21-23]. Furthermore, the degree of metabolism with regard to *NAT2* varies among individuals, suggesting that genetic variations contribute to the metabolic activation capacity. Although studies on the association between *NAT2* phenotype (slow acetylator)[24] and anti-TB drug-induced hepatotoxicity have been reported from Taiwan[15,18], India[6,16], and Japan[13,14,17], no study has examined the association between hepatotoxicity and haplotypes/diplotypes that are composed of single nucleotide polymorphisms (SNPs). In the present study, we report our findings of the association between *NAT2* haplotypes/diplotypes and anti-TB drug-induced adverse effects, especially hepatotoxicity, in Japanese TB patients.

MATERIALS AND METHODS

Subjects

The study subjects comprised of 100 patients with new onset of pulmonary TB treated with a INH- (400 mg/d) and rifampicin (RFP, 450 mg/d)-containing regimen for six or nine months, between the years of 2003 and 2005 (Table 1). All subjects were Japanese who were recruited randomly from four general health clinics in the Nagasaki area of Japan. The study protocol was approved by the Committee for the Ethical Issue on Human Genome and Gene Analysis in Nagasaki University, and written informed consent was obtained from each patient.

The diagnosis of pulmonary TB was made on the basis of symptoms, chest radiographic infiltrates, and presence of acid-fast bacilli on sputum smear and *M. tuberculosis* on sputum culture. Patients with liver cirrhosis, chronic and acute hepatitis, alcoholic liver disease, and other chronic liver diseases were excluded from the study.

Table 1 Characteristics of pulmonary TB patients included in the study

Characteristics	TB
Number of patients	100
Age range (yr)	22-94
Age (mean ± SD)	64.0 ± 17.4
Gender (male/female)	56/44
Body mass index (kg/m^2)	20.3 ± 2.9

Diagnosis of drug-induced adverse effects

Patients with TB were classified into the following two subgroups: those with adverse effects such as hepatotoxicity, skin rash, and eosinophilia, and those without any side effects. Drug-induced hepatotoxicity was defined according to the criteria of the International Consensus Meeting[25], i.e., development of a two-fold or more increase in serum alanine aminotransferase (ALT) level above the upper limit of the normal range: N ($\leqslant$ 42 IU/L), or a combined increase of over 2 N in serum aspartate aminotransferase (AST, $N \leqslant$ 33 IU/L) and total bilirubin (TB, $N \leqslant$ 1.5 mg/dL). The presence of > 450 eosinophils/mL was defined as eosinophilia.

Determination of NAT2 polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes of each patient using the DNA Extracter WB-Rapid Kit (Wako, Osaka, Japan), according to the manufacturer's protocol. SNPs of *NAT2* deposited in the SNP-database[26] were determined with PCR-restriction fragment length polymorphism (RFLP) method as described previously[27,28]. PCR was performed in a 25-μL reaction mixture containing 20 ng of genomic DNA, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 0.4 μmol/L each of sense and antisense primers, and 1.5 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) with a DNA thermal cycler, GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), according to the following protocol: initial denaturation at 95℃ for 5 min; 35 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s; and a final extension at 72℃ for 5 min. Subsequently, the PCR product was digested by restriction enzyme (TaKaRa Biomedical, Shiga, Japan) for detection of each SNP. A SNP, C282T, was detected by digestion with *Fok* I. Likewise, C481T, G590A, and G857A were detected by *Kpn* I, *Taq* I, or *Bam* HI, respectively. These fragments were subjected to electrophoresis on a 2% agarose gel, and visualized with UV transilluminator (Alpha Innotech, San Leandro, CA, USA) after ethidium bromide (Invitrogen) staining. Haplotypes were determined to be based on a combination of four SNPs (Table 2)[26,28].

Statistical analysis

Data obtained are shown as mean ± standard deviation (SD). Clinico-pathological parameters were compared between TB patients with and without adverse effect, using the Mann-

Table 2 Five Haplotypes composed of four SNPs in *NAT2*

Haplotype	SNP			
	C282T	C481T	G590A	G857A
*NAT2*4*	-	-	-	-
*NAT2*6A*	+	-	+	-
*NAT2*7B*	+	-	-	+
*NAT2*11*	-	+	-	-
*NAT2*13*	+	-	-	-

Plus or minus symbols for C282T, C481T, G590A, and G857A indicate the presence or absence of SNPs.

Whitney *U* test, χ^2 test with Yates' correction, and Fisher's exact test. Expected allele frequencies were calculated from respective single allele frequencies according to the Hardy-Weinberg equilibrium. The observed and expected allele frequencies were compared by a χ^2 test using SNP Alyze 6.0 standard (Dynacom Inc., Chiba, Japan). To evaluate odds ratio (OR) with 95% confidence interval (95% CI) for the susceptibility to anti-TB drug-induced adverse effects, haplotype and diplotype frequencies were compared between TB patients with and without adverse effects, using multivariate logistic regression analysis. A *P* value of 0.05 or less was considered statistically significant. SPSS 14.0 (SPSS Japan Inc., Tokyo, Japan) program package was used for all statistical analyses.

RESULTS

Frequency of drug-induced adverse effects, and clinico-pathological parameters for susceptibility to the effects

Out of the 100 TB patients enrolled in the study, 50 (50%) patients had anti-TB drug-induced adverse effects, 18 hepatotoxicity, 25 skin rash, and 34 eosinophilia. There were no differences in the clinical characteristics and baseline laboratory data (before chemotherapy) between TB patients with and without adverse effects (Table 3). However, eosinophilia developed less frequently in female patients than male patients ($P = 0.0186$). During TB chemotherapy, patients with hepatotoxicity had 8-times higher serum levels of ALT and AST than those without hepatotoxicity ($P < 0.0001$). Likewise, during therapy, ALT values and eosinophil counts were significantly higher in patients with skin rash compared to those without skin rash ($P = 0.0245$ and $P = 0.0058$, respectively). Moreover, eosinophils in patients with eosinophilia were increased in number compared with those without this complication ($P < 0.0001$).

NAT2-haplotype susceptible to adverse effects

In the 100 TB patients examined, we identified three haplotypes composed of four SNPs (Table 4). One haplotype, "*NAT2*4*" is a wild-type (major type), while the other haplotypes are variants (minor types). Distribution of SNPs and haplotypes among patients corresponded well with the Hardy-Weinberg equilibrium, implying that our samples had a homogeneous genetic background, and was consistent with previous observations[13,14,17]. However, since the frequencies of two haplotypes, *NAT2*11* and *NAT2*13*, were very low, they were not used for further statistical analysis.

Multivariate logistic regression analyses revealed that the frequency of a variant haplotype "*NAT2*6A*", which is composed of two SNPs (C282T and G590A), was significantly increased in TB patients with hepatotoxicity, compared with those without hepatotoxicity ($P = 0.001$, OR = 3.535, 95% CI: 1.648-7.585) (Table 4). By contrast, the frequency of the wild-type (major) haplotype, "*NAT2*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity ($P < 0.001$, OR = 0.265). There were no significant differences in the frequency of *NAT2*-haplotypes between TB patients with and without skin rash or eosinophilia (Table 4).

NAT2-diplotype susceptible to adverse effects

We identified six diplotypes composed of three haplotypes (Table 5). Distributions of the diplotypes in our study population were consistent with previous observations [13,14,17]. Of a total of 18 TB patients with hepatotoxicity, 3 (16.6%) had a diplotype, "*NAT2*6A/*7B*"; using multivariate logistic regression analyses, the frequency was significantly higher than in patients without hepatotoxicity (2/82, 2.4%; $P = 0.029$, OR = 8.000, 95% CI: 1.230-52.023) (Table 5). On the other hand, the frequency of another diplotype, "*NAT2*4/*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity ($P = 0.032$, OR = 0.272). There was no difference in the frequency of *NAT2*-diplotypes between TB patients with and without skin rash or eosinophilia (Table 5).

DISCUSSION

We have shown that a variant haplotype, *NAT2*6A*, of *NAT2* is associated with susceptibility to anti-TB drug-induced hepatotoxicity, and a wild-type (major) haplotype, *NAT2*4*, is associated with non-susceptibility to hepatotoxicity. These findings suggest that *NAT2* is one of the genetic factors responsible for predisposition to anti-TB drug-induced hepatotoxicity. However, since the number of TB patients in the present study was relatively small, it remains to be confirmed whether this association can be reproduced in a larger number of Japanese TB patients with and without hepatotoxicity as well as in other ethnic populations. Although previous reports have shown a positive association in Japanese TB patients between drug-induced hepatotoxicity and *NAT2* variants with phenotypic activities of *NAT2*, such as rapid, intermediate, and slow acetylators[13,14,17], the present study is the first report demonstrating an association with *NAT2*-haplotype variation.

Three *NAT2* haplotypes, *NAT2*5B*, *NAT2*6A*, and *NAT2*7B*, are believed to be associated with slow acetylators[24,29,30]. We did not detect *NAT2*5B* in our samples, probably because of its low frequency in the Japanese population as described in our previous study[28]. Since *NAT2* is the main enzyme involved in the metabolism of INH and *NAT2*6A* is functionally related to the low activity of *N*-acetylation in the INH metabolic pathway[30], TB patients possessing *NAT2*6A* may fail to metabolize toxic substances, such as hydrazine and acetylhydrazine, generated by INH metabolism in the liver, which therefore accumulate in the body, leading to drug-induced hepatotoxicity[21-23,31,32].

Table 3 Clinical characteristics and laboratory data of TB patients with or without adverse effect

Clinical data	Hepatotoxicity		*P*	Skin rash		*P*	Eosinophilia		*P*
	Present (*n* = 18)	Absent (*n* = 82)		Present (*n* = 25)	Absent (*n* = 75)		Present (*n* = 34)	Absent (*n* = 66)	
Age (mean ± SD)	60.8 ± 17.7	64.7 ± 17.3	0.3942	63.6 ± 18.1	64.7 ± 17.3	0.9028	63.3 ± 19.5	64.4 ± 16.3	0.7598
Gender (M/F)	9/9	47/35	0.6081	12/13	44/31	0.3640	25/9	31/35	0.0186
Body mass index (kg/m^2)	19.6 ± 2.3	20.5 ± 3.1	0.2721	19.8 ± 2.5	20.6 ± 3.1	0.2626	19.8 ± 2.9	20.6 ± 3.0	0.2684
Baseline values									
ALT (IU/L)	18.0 ± 10.4	21.1 ± 16.6	0.4527	23.1 ± 25.8	19.7 ± 10.4	0.3392	25.9 ± 24.4	17.8 ± 6.8	0.6571
AST (IU/L)	29.1 ± 26.8	26.8 ± 23.3	0.7188	31.3 ± 39.2	25.9 ± 15.9	0.3268	35.4 ± 38.5	23.0 ± 7.8	0.4277
TB (mg/dL)	0.48 ± 0.19	0.64 ± 0.43	0.1115	0.61 ± 0.44	0.61 ± 0.39	0.9874	0.62 ± 0.44	0.61 ± 0.38	0.9490
Creatinine (mg/dL)	0.64 ± 0.13	0.88 ± 1.10	0.3482	0.72 ± 0.28	0.87 ± 1.13	0.5121	0.82 ± 0.47	0.84 ± 1.17	0.9092
Eosinophils (/μL)	105.1 ± 120.6	115.5 ± 121.8	0.7434	104.7 ± 93.7	116.6 ± 129.3	0.6750	141.3 ± 133.4	99.3 ± 112.6	0.1011
During TB chemotherapy									
Peak ALT (IU/L)	316.2 ± 281.7	40.0 ± 19.5	< 0.0001	147.8 ± 281.7	61.6 ± 88.1	0.0245	129.6 ± 284.1	59.2 ± 75.4	0.3885
Peak AST (IU/L)	294.5 ± 353.6	36.7 ± 21.5	< 0.0001	139.7 ± 222.9	73.1 ± 128.9	0.1325	116.3 ± 175.9	76.0 ± 149.3	0.2107
Peak TB (mg/dL)	1.20 ± 1.16	0.74 ± 0.53	0.0101	0.78 ± 0.47	0.84 ± 0.77	0.7039	0.92 ± 0.89	0.77 ± 0.58	0.3241
Peak Creatinine (mg/L)	0.76 ± 0.13	0.96 ± 1.23	0.4742	0.81 ± 0.13	0.97 ± 1.28	0.5316	0.87 ± 0.31	0.96 ± 1.36	0.7098
Peak Eosinophils (/μL)	692.4 ± 929.1	461.1 ± 844.7	0.4538	668.4 ± 773.9	447.5 ± 885.1	0.0058	1028.1 ± 1327.9	232.1 ± 114.9	< 0.0001

Table 4 Distributions of *NAT2*-haplotypes in TB patients with and without adverse effect

Haplotype	Hepatotoxicity					Skin rash					Eosinophilia				
	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*
*NAT2*4*	16 (44.4)	120 (73.2)	0.265	0.129-0.546	< 0.001	34 (68.0)	102 (68.0)	1.00	0.503-1.987	1.000	45 (66.2)	91 (68.9)	0.880	0.472-1.642	0.688
*NAT2*6A*	14 (38.9)	29 (17.7)	3.535	1.648-7.585	0.001	12 (24.0)	31 (20.7)	1.22	0.570-2.607	0.609	15 (22.0)	28 (21.2)	1.052	0.517-2.138	0.889
*NAT2*7B*	6 (16.7)	15 (9.1)	2.235	0.818-6.104	0.117	4 (8.0)	17 (11.3)	0.70	0.226-2.170	0.537	8 (11.8)	13 (9.9)	1.227	0.482-3.124	0.667
Total number	36	164				50	150				68	132			

Table 5 Distribution of *NAT2*-diplotypes in TB patients with and without adverse effect

Diplotype	Hepatotoxicity					Skin rash					Eosinophilia				
	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*
*NAT2*4/*4*	4 (22.2)	42 (51.2)	0.272	0.083-0.897	0.032	12 (48.0)	34 (45.3)	1.113	0.449-2.757	0.817	14 (41.2)	32 (48.5)	0.744	0.322-1.717	0.488
*NAT2*4/*6A*	7 (38.9)	23 (28.1)	1.632	0.564-4.726	0.366	7 (28.0)	23 (30.7)	0.879	0.323-2.394	0.801	11 (32.4)	19 (28.9)	1.183	0.484-2.894	0.713
*NAT2*4/*7B*	1 (5.6)	13 (15.9)	0.312	0.038-2.555	0.278	3 (12.0)	11 (14.7)	0.793	0.203-3.108	0.740	6 (17.6)	8 (12.1)	1.554	0.492-4.909	0.453
*NAT2*6A/*6A*	2 (11.1)	2 (2.4)	5.000	0.655-38.152	0.121	2 (8.0)	2 (2.7)	3.174	0.423-23.812	0.261	1 (2.9)	3 (4.5)	0.636	0.064-6.360	0.700
*NAT2*6A/*7B*	3 (16.6)	2 (2.4)	8.000	1.230-52.023	0.029	1 (4.0)	4 (5.3)	0.74	0.079-6.945	0.792	2 (5.9)	3 (4.5)	1.313	0.209-8.257	0.772
*NAT2*7B/*7B*	1 (5.6)	0 (0)	-	-	-	0 (0)	1 (1.3)	-	-	-	0 (0)	1 (1.5)	-	-	-
Total number	18	82				25	75				34	66			

A variant diplotype, *NAT2*6A/*7B*, is associated with susceptibility to hepatotoxicity ($P = 0.029$). Although another *NAT2*-diplotype, *NAT2*6A/*6A*, showed a trend towards susceptibility to hepatotoxicity, the results were statistically not significant ($P = 0.121$). However, if a larger number of subjects were analyzed, *NAT2*6A/*6A* as well as *NAT2*6A/*7B* may demonstrate a significant association with hepatotoxicity. Both of these diplotypes are homozygous for variant haplotypes and indicate phenotypically slow acetylators. Therefore, it is likely that some of the slow acetylators who are variant homozygotes possessing the *NAT2*6A* haplotype have susceptibility to anti-TB drug-induced hepatotoxicity. In this context, the results of the present study with regard to *NAT2*-haplotypes/diplotypes are comparable to those of previous reports on the association between *NAT2* phenotypic variation and hepatotoxicity[13-17,32]. Conversely, a wild-type homozygote, *NAT2*4/*4*, is associated with non-susceptibility and resistance to hepatotoxicity.

In conclusion, the haplotypes, *NAT2*4* and *NAT2*6A*, are new biomarkers for predicting drug-induced hepatotoxicity, and may prove useful in achieving optimal treatment of individual TB patients.

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COMMENTS

Background

Tuberculosis (TB) is a re-emerging infectious disease and has been declared a global health problem by the WHO. Adverse effect of anti-TB drugs including

isoniazid (INH) has become a serious problem in the management of tuberculosis. Risk factors associated with the development of adverse effects include both clinical and genetic factors. Recently, genome-wide screening and candidate gene-based association studies have been launched to identify the possible susceptibility genes sensitive to anti-TB drugs.

Research frontiers

Association studies with candidate gene-based approach in Asian and Caucasian patients have identified several possible susceptibility genes, e.g., *N*-acetyltransferase 2 (*NAT2*), cytochrome P450 2E1, glutathione *S*-transferase M1, glutathione *S*-transferase T1, and HLA-DQA1/-DQB1.

Innovations and breakthroughs

There are several reports on the association between *NAT2* polymorphisms and adverse effects, especially hepatotoxicity, of anti-TB drugs from Japan, Taiwan, and India. However, *NAT2* polymorphisms have been analyzed as phenotypic activities of *NAT2*, such as rapid, intermediate, and slow acetylators, but not as *NAT2*-haplotypes. The present study has shown that some phenotypically slow acetylators who are variant homozygotes possessing *NAT2*6A* haplotype have increased susceptibility to anti-TB drug-induced hepatotoxicity. This is the first report on the association with *NAT2*-haplotypes and hepatotoxicity in Japanese TB patients.

Applications

Our findings can be used for DNA-based diagnosis of TB patients before initiating treatment with anti-TB drugs, using *NAT2*6A* as a biomarker. Since patients possessing *NAT2*6A* haplotype have higher susceptibility to anti-TB-drug-induced hepatotoxicity, such individuals should be treated by reducing the dose of INH from 400 to 200 mg, in order to achieve optimal results.

Terminology

NAT2 is the main enzyme in the INH metabolism, and is expressed in the liver. Single nucleotide polymorphism (SNP) is a DNA sequence variation which occurs when a single nucleotide in the genome differs in paired chromosomes of an individual. Haplotype is a combination of alleles at multiple linked loci that are transmitted together. A second interpretation is that a haplotype is a set of SNPs on a single chromatid that is statistically associated. Such information is very valuable in investigating the genetics behind common diseases. Restriction fragment length polymorphism (RFLP) is a laboratory technique designed to distinguish differing nucleotide sequences from two related contexts.

Peer review

This study is well performed and the subject matter is very interesting.

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CLINICAL RESEARCH

Risk factors for gastroesophageal reflux disease, reflux esophagitis and non-erosive reflux disease among Chinese patients undergoing upper gastrointestinal endoscopic examination

Juan Du, Jiang Liu, Hong Zhang, Chao-Hui Yu, You-Ming Li

Juan Du, Jiang Liu, Hong Zhang, Chao-Hui Yu, You-Ming Li, Department of Gastroenterology, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
Correspondence to: You-Ming Li, Department of Gastroenterology, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China. zlym@zju.edu.cn
Telephone: +86-571-87236603 Fax: +86-571-87236611
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Abstract

AIM: To analyze the spectrum and risk factors of gastroesophageal reflux disease (GERD) based on presenting symptoms and endoscopic findings.

METHODS: A cross-sectional survey in a cluster random sample was conducted from November 2004 to June 2005 using a validated Chinese version Reflux Disease Questionnaire (RDQ) and other items recording the demographic characteristics and potential risk factors for GERD. Subjects were defined as having GERD symptoms according to the RDQ score (> 12). All subjects were endoscopied and the definition and severity of erosive esophagitis were evaluated by Los Angeles classification. The statistical analysis was performed with SPSS13.0 programs.

RESULTS: Of 2231 recruited participants, 701 (31.40%) patients were diagnosed as having GERD while 464 (20.80%) patients had objective findings of reflux esophagitis (RE). Of those 464 patients, only 291 (13.00%) were reported as subjects with GERD symptoms. A total of 528 (23.70%) patients were found to have GERD symptoms, including 19.50% patients with grade A or B reflux esophagitis, 0.90% with grade C and 0.40% with grade D. On multivariate analysis, old age, male, moderate working burden, divorced/widowed and strong tea drinking remained as significant independent risk factors for erosive esophagitis. Meanwhile, routine usage of greasy food and constipation were considered as significant independent risk factors for non-erosive reflux disease (NERD).

CONCLUSION: GERD is one of the common GI diseases with a high occurrence rate in China and its main associated factors include sex, anthropometrical variables and sociopsychological characteristics.

Key words: Gastroesophageal reflux disease; Reflux esophagitis; Non-erosive reflux disease; Prevalence; Risk factors; Endoscopy

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disorder with a high incidence rate of 10%-38% of adults in the Western population occurring at least once a week[1,2]. The prevalence of GERD has been increasing[3]. The diagnosis and treatment of GERD are therefore, important because the disease, in addition to the highly disturbing typical symptoms, has a series of known consequences. The presence of GERD may affect the patients' quality of life[4], decrease functional activity[5], increase the economic burden[6] and the risk of esophageal carcinoma in the cases of Barrett's esophagus[7]. With an emphasis on morphological diagnosis, endoscopy has become a major tool to assess the final consequences of GERD, which is especially useful for population-based screening.

Although many investigators have reported the prevalence of erosive esophagitis[8], the prevalence of NERD has not been investigated in China. Our study was designed to analyze the spectrum of GERD subjects based on presenting symptoms and endoscopic findings. In order to determine the risk factors for such disease in outpatients from Zhejiang Province of East China, a cross-sectional survey in a cluster random sample was conducted from November 2004 to June 2005.

MATERIALS AND METHODS

Subjects

From November 2004 to June 2005, outpatients visiting departments of medicine in 10 hospitals from island, mountainous area, plain, city, countryside and suburb, in the Zhejiang Province of East China were recruited to the study. Subjects were excluded if they were not permanent inhabitants of East China, less than 18 years old, and had major psychotic episodes, mental retardation, dementia, severe visual or hearing abnormalities or other illnesses that might render them unable to complete the questionnaire or undergo the endoscopy (e.g. stroke). Excluding criteria also contained a history of peptic ulcer disease and receiving proton pump inhibitors or H_2-blockers over the preceding 2 wk. A total of 2278 individuals who had GI endoscopy were recruited, of which 2231 were eligible with a response rate of 97.9%.

Questionnaire

The gastroesophageal reflux questionnaire, a self-report instrument that to evaluate reflux-associated symptoms during the prior month, included the Chinese version of the Reflux Diagnostic Questionnaire (RDQ)[9] and items concerning the demographic characteristics and probable risk factors for GERD. It comprised the following parts: (1) General information: gender and age. (2) The Chinese version of the Reflux Diagnostic Questionnaire (RDQ): its framework of the RDQ was based on a validated questionnaire previously published[3]. The Chinese version of the RDQ was designed to measure symptoms suggestive of GERD appearing during the previous month. The intraclass correlation coefficient of the Chinese version of the RDQ was 0.9, thus it was validated and found to be a useful screening test for GERD-associated symptoms in China[9]. The symptoms suggestive of GERD in the RDQ included heartburn, substernal chest pain, acid eructation and food regurgitation. The following definitions were used to identify the symptoms in the RDQ: (1) heartburn, a burning sensation located beneath the sternum; (2) substernal chest pain: any pain felt inside in the chest but not including heartburn or any pain that is primarily originated from the abdomen; (3) acid regurgitation, a bitter- or sour-tasting fluid coming into the throat or mouth; and (4) food regurgitation, unpleasant movement of material upwards from the stomach but not vomit. Each symptom was scored according to the frequency and severity (5-point scale). The highest score for one subject was 40. The frequency was measured according to the following scale: 0, no symptom in the past month; 1, less than once a week; 2, once a week; 3, two to three days a week; 4, four to five days a week; and 5, almost daily. Symptom severity was assessed on the following scale:0, none; 1, very mild (symptoms can be easily ignored unless reminded of them); 2, mild (between 1 and 3); 3, moderate (symptoms are obvious and sufficient to influence normal activities, and occasionally need treatment); 4, severe (between 3 and 5); and 5, very severe (symptoms are obvious and sufficient to influence normal activities, and need long-term medication).

The Chinese version of the RDQ has been tested in a multicenter study including 10 hospitals in China. The specificity and sensitivity of the RDQ were evaluated by comparing the results with those of upper gastrointestinal endoscopy and esophageal 24-h pH monitoring. The RDQ score correlated positively with the severity of reflux esophagitis. Esophageal pH monitoring showed that patients with abnormal RDQ scores had higher Demeester scores than those with normal RDQ scores (20.18 *vs* 16.84). Taking 12 as the parameter for the threshold of RDQ score for GERD-associated symptoms, the study group obtained the maximal Youden index, the area under the receiver operating characteristic curve (ROC), was 0.71, the true positive diagnostic rate was 88.07% and the true negative diagnostic rate was 68.42% with a sensitivity of 94.12% and specificity of 50.00%. The subject was defined as a patient with GERD symptoms if his/her RDQ score was higher than 12[9]. Probable risk factors for GERD symptoms included life status: working burden, marital status (married, single, divorced/widowed), constipation, dietary and other personal habits: excessive consumption of acidic beverages, coffee, strong tea, spicy food, greasy food, sweet food, cigarette and alcohol. Definitions: heavy smoker (more than 20 cigarettes per day), excessive alcohol ($\leqslant$ 210 g of alcohol per week), constipation (frequently occurred during last 12 mo), routine use of coffee (more than 100 mL per day on average), acidic beverages and strong tea (more than 200 mL per day on average), dietary habits (taking the food mentioned above more than one time per day on average). The questions about probable risk factors, extra esophageal symptoms and accompanying diseases were all binary: yes or no.

Upper gastrointestinal endoscopy

Patients were examined for the presence of reflux esophagitis. Diagnosis and classification of reflux esophagitis were based on the Los Angeles classification[8]. Barrett's esophagus was diagnosed when columnar epithelium was seen to extend the Z line and confirmed histologically that showed specialized intestinal metaplasia. These criteria were consistently applied and endoscopic diagnosis was confirmed by either of the authors who were present during each endoscopic procedure. Patients who returned for endoscopic reassessment for any reason were excluded from the analysis to prevent duplication of cases.

Survey design and response rate

The cross-sectional survey in a cluster random sample was conducted from November 2004 to June 2005. The present study was based on a standard protocol including routine internal medicine counseling, endoscopy, and a self-reported questionnaire. Consecutive numbers were assigned to each registered subject and a 1:10 ratio of sample was selected using random number tables. All subjects completed the detailed questionnaire before endoscopy. Confirmed consent was obtained from all patients before the questionnaire was administered. All subjects were given the questionnaire.

A subject with GERD symptoms was defined according to the RDQ score (> 12). Patients who were

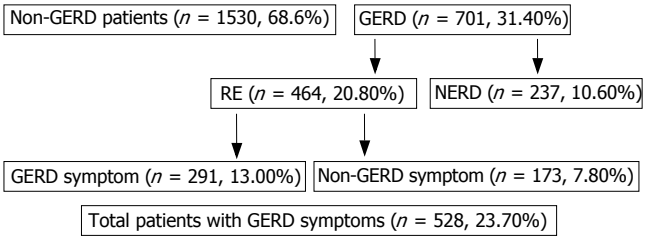

Figure 1 Diagnosis of gastroesophageal reflux disease (GERD) based on symptoms and presence of reflux esophagitis (RE).

Table 1 Grading of reflux esophagitis in 2278 patients

Grade	Total number	%
None	1767	79.20
A	333	14.93
B	102	4.57
C	20	0.90
D	9	0.40
Total	2231	100.00

Table 2 Complications of reflux esophagitis

	Total number	%
Barrett's esophagus	No 1190	98.76
	Yes 15	1.24
Esophageal stenosis	No 1191	98.76
	Yes 15	1.24
Esophagorrhagia	No 1186	98.18
	Yes 22	1.82

suspicious of having GERD but without evidence of reflux esophagitis (RE) were diagnosed as having NERD. GERD was diagnosed based on the presence of reflux esophagitis and/or the presence of predominant reflux symptoms. Because the survey explanation is made according to the RDQ score, all the questions in the RDQ must be answered without omission. In this study, a total of 2231 eligible subjects were recruited.

Ethics

The study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

Statistical analysis

The database was established with Epidata3.0. Statistical analysis was performed with SPSS13.0 programs. Univariate analysis was performed using χ^2 test for categorical variables. Univariate and multivariate logistic regression models were used to identify the potential risk factors of GERD, NERD and reflux esophagitis. The probable risk factors for GERD symptoms were selected by the univariate logistic regression, including life status (labor burden, marital status), dietary and other personal habits such as routine usage of acidic beverage, spicy food, greasy food, coffee, strong tea, sweet food, cigarette and alcohol, and constipation. All risk factors associated with GERD symptoms on univariate analysis were modeled using multivariate forward stepwise logistic regression analysis. To find the best model, a forward elimination stepwise procedure was carried out in a way that the factor would be brought into the analysis if the corresponding P value was less than 0.5. A P value $\geqslant 0.05$ was considered statistically significant and all P values were obtained by two-tailed examination.

Table 3 Comparison between upper gastrointestinal endoscopy and RDQ (χ^2 test)

RDQ	Endoscopy		*P* value
	Negative	Positive	
Negative	1530	173	0.002
Positive	237	291	

$P = 0.002$, there is significant statistical difference between the two investigations.

Table 4 Comparison between upper gastrointestinal endoscopy and RDQ (Wilcoxon rank-sum test)

RDQ	Endoscopy					Total	*U* value	*P* value
	Negative	A	B	C	D			
Negative	1530	131	34	5	3	1703		
Positive	237	202	68	15	6	528	3.60	0.000
Total	1767	333	102	20	9	2231		

RESULTS

Sample characteristics

A total of 2231 (56.6% male, 43.4% female) outpatients aged from 18 to 90 years (a median age of 43 years) were recruited to this study.

Prevalence of GERD, reflux esophagitis and NERD

As shown in Figure 1, 701 (31.40%) patients were diagnosed as having GERD and 464 (20.80%) patients were found to have objective findings of reflux esophagitis. Of the 464 patients, only 291 (13.00%) presented with GERD symptoms. Among the 2231 subjects, 528 (23.70%) presented with GERD symptoms.

Distribution of different grades and complications of reflux esophagitis

As shown in Table 1, 435 patients (19.50%) had grade A or B reflux esophagitis while 20 patients had grade C (0.90%) and 9 had grade D (0.40%). The complications of reflux esophagitis are shown in Table 2. The most frequent complication is esophagorrhagia (1.82%).

Comparison between upper gastrointestinal endoscopy and RDQ

As shown in Tables 3 and 4, there is significant difference between the two investigations. The Kappa value was 0.47, $P = 0.000$, demonstrating no predominant consistency between the two diagnostic methods.

Risk factors in GERD and non-GERD patients

The prevalence of various variables in GERD patients

Table 5 Association between variables determined using univariate analysis: GERD (n = 701) *vs* non-GERD (n = 1530) patients

Variables	GERD n (%)	P value	OR	Univariate (95% CI)	
				Low	High
Age (yr)					
> 65	106/239 (44.40)	< 0.0001	0.53	0.41	0.70
≤ 65	595/1992 (29.90)				
Gender					
Male	439/1263 (34.80)	< 0.0001	0.7	0.58	0.84
Female	262/968 (27.10)				
Working burden					
Heavy	64/147 (43.50)	0.694	1.04	0.86	1.27
Moderate	299/975 (30.70)	0.004	0.6	0.42	0.85
Mild	282/895 (31.50)				
Marital status					
Divorced/ widowed	6/14 (42.90)	< 0.0001	2.05	1.49	2.82
Single	52/261 (19.90)	0.478	0.68	0.24	1.97
Married	592/1752 (33.80)				
Excessive eating					
Yes	245/704 (34.80)	0.009	0.77	0.63	0.94
No	372/1276 (29.20)				
Routine intake of greasy food					
Yes	231/618 (37.40)	< 0.0001	0.65	0.53	0.80
No	375/1347 (27.80)				
Routine intake of spicy food					
Yes	196/512 (38.30)	< 0.0001	0.65	0.52	0.80
No	418/1463 (28.60)				
Routine intake of acidic beverage					
Yes	118/358 (33.00)	0.403	0.90	0.71	1.15
No	493/1606 (30.70)				
Routine intake of strong tea					
Yes	149/343 (43.40)	< 0.0001	0.51	0.4	0.64
No	451/1613 (28.00)				
Routine intake of sweet food					
Yes	225/683 (32.90)	0.122	0.85	0.70	1.04
No	375/1269 (29.60)				
Heavy smoking					
Yes	209/562 (37.20)	< 0.0001	0.68	0.55	0.83
No	407/1426 (28.50)				
Excessive alcohol					
Yes	185/503 (36.80)	0.002	0.71	0.57	0.88
No	434/1486 (29.20)				
Routine intake of coffee					
Yes	26/77 (33.80)	0.529	0.86	0.53	1.39
No	568/1869 (30.40)				
Constipation					
Yes	122/316 (38.60)	0.002	0.68	0.53	0.87
No	489/1641 (29.80)				

GERD: Gastroesophageal reflux disease.

Table 6 Association between variables determined using univariate analysis: Reflux esophagitis (n = 464) *vs* non-GERD (n = 1530) patients

Variables	RE n (%)	P	OR	Univariate (95% CI)	
				Low	High
Age (yr)					
> 65	77/210 (36.70)	< 0.0001	2.09	1.55	2.82
≤ 65	387/1784 (21.70)				
Gender					
Male	322/1146 (28.10)	< 0.0001	1.94	1.56	2.43
Female	142/848 (16.70)				
Working burden					
Heavy	45/128 (35.20)	0.353	0.90	0.71	1.13
Moderate	189/865 (21.80)	0.006	1.74	1.17	2.59
Mild	191/804 (23.80)				
Marital status					
Divorced/ widowed	5/13 (38.50)	< 0.0001	0.48	0.33	0.70
Single	34/243 (14.00)	0.283	1.85	0.60	5.69
Married	392/1552 (25.30)				
Excessive eating					
Yes	147/606 (24.30)	0.346	1.12	0.89	1.41
No	259/1163 (22.30)				
Routine intake of greasy food					
Yes	129/516 (25.00)	0.090	1.23	0.97	1.57
No	263/1235 (21.30)				
Routine intake of spicy food					
Yes	112/428 (26.20)	0.037	1.31	1.02	1.69
No	283/1328 (21.30)				
Routine intake of acidic beverage					
Yes	76/316 (24.10)	0.593	1.08	0.81	1.44
No	326/1439 (22.70)				
Routine intake of strong tea					
Yes	108/302 (35.80)	< 0.0001	2.28	1.74	2.98
No	284/1446 (19.60)				
Routine intake of sweet food					
Yes	131/589 (22.20)	0.769	0.97	0.76	1.22
No	265/1159 (22.90)				
Heavy smoking					
Yes	153/506 (30.20)	< 0.0001	1.75	1.38	2.22
No	252/1271 (19.80)				
Excessive alcohol					
Yes	124/442 (71.90)	0.003	1.45	1.13	1.85
No	283/1335 (21.20)				
Routine intake of coffee					
Yes	21/72 (29.20)	0.167	1.44	0.86	2.43
No	371/1672 (22.20)				
Constipation					
Yes	67/261 (25.70)	0.275	1.18	0.87	1.60
No	336/1488 (22.60)				

GERD: Gastroesophageal reflux disease.

compared to non-GERD patients is shown in Table 5. On univariate analysis, age > 65, male, moderate working burden, divorced/widowed, excessive eating, greasy food, spicy food, strong tea, smoking, alcohol, and constipation were found to be significant participants. On multivariate analysis, old age (OR, 0.57; β, -0.57; 95% CI, 0.40-0.80), male (OR, 0.78; β, -0.25; 95% CI, 0.61-1.00), moderate working burden (OR, 0.58; β, -0.54; 95% CI, 0.39-0.87), divorced/widowed (OR, 1.82; β, 0.60; 95% CI, 1.27-2.60), greasy food (OR, 0.75; β, -0.29; 95% CI, 0.60-0.95), strong tea (OR, 0.67; β, -0.40; 95% CI, 0.50-0.89) remained as significant independent risk factors.

Risk factors in reflux esophagitis and non-GERD patients

The prevalence of various variables in reflux esophagitis patients compared to non-GERD patients is shown in Table 6. On univariate analysis, age > 65, male, moderate working burden, divorced/widowed, spicy food, strong tea, smoking, and alcohol were found to be significant. On multivariate analysis, old age (OR, 1.86; β, 0.62; 95% CI, 1.29-2.70), male (OR, 1.77; β, 0.57; 95% CI, 1.32-2.37), moderate working burden (OR, 1.91; β, 0.65; 95% CI, 1.22-2.97), divorced/widowed (OR, 0.55; β, -0.60; 95% CI, 0.36-0.85), strong tea (OR, 1.62; β, 0.48; 95% CI, 1.18-2.23) were considered as significant independent risk factors.

Risk factors in NERD and non-GERD patients

The prevalence of various variables in NERD patients compared to non-GERD patients is shown in Table 7. On univariate analysis, divorced/widowed, excessive eating,

Table 7 Association between variables determined using univariate analysis: NERD (*n* = 237) *vs* non-GERD (*n* = 1530) patients

Variables	NERD *n* (%)	*P*	OR	Univariate (95% CI)	
				Low	High
Age (yr)					
> 65	29/162 (17.90)	0.08	1.46	0.96	2.25
≤ 65	208/1605 (13.00)				
Gender					
Male	117/941 (12.40)	0.198	0.84	0.64	0.10
Female	120/826 (14.50)				
Working burden					
Heavy	19/102 (8.60)	0.547	1.10	0.81	1.48
Moderate	110/786 (14.00)	0.119	1.54	0.89	2.66
Mild	91/704 (12.90)				
Marital status					
Divorced/ widowed	1/9 (11.10)	0.007	0.5	0.30	0.83
Single	18/227 (7.90)	0.762	0.73	0.09	5.83
Married	200/1360 (14.70)				
Excessive eating					
Yes	98/557 (17.60)	< 0.0001	1.71	1.27	2.29
No	113/1017 (11.10)				
Routine intake of greasy food					
Yes	102/489 (20.90)	< 0.0001	2.29	1.71	3.07
No	112/1084 (10.30)				
Routine intake of spicy food					
Yes	84/400 (21.00)	< 0.0001	2.06	1.52	2.78
No	135/1180 (11.40)				
Routine intake of acidic beverage					
Yes	42/282 (14.900	0.41	1.17	0.81	1.68
No	167/1280 (13.00)				
Routine intake of strong tea					
Yes	41/235 (17.40)	0.043	1.47	1.01	2.14
No	167/1329 (12.60)				
Routine intake of sweet food					
Yes	94/552 (17.00)	0.001	1.67	1.24	2.25
No	110/1004 (11.00)				
Heavy smoking					
Yes	56/409 (13.70)	0.802	1.04	0.75	1.45
No	155/1174 (13.20)				
Excessive alcohol					
Yes	61/379 (16.10)	0.078	1.34	0.97	1.85
No	151/1203 (12.60)				
Routine intake of coffee					
Yes	5/56 (8.90)	0.36	0.65	0.26	1.64
No	197/1498 (13.20)				
Constipation					
Yes	55/249 (22.10)	< 0.001	2.14	1.51	3.01
No	153/1305 (11.70)				

GERD: Gastroesophageal reflux disease; NERD: Non-erosive reflux disease.

greasy food, spicy food, strong tea and constipation were found to be significant. On multivariate analysis, greasy food (OR, 1.65; β, 0.50; 95% CI, 1.16-2.36) and constipation (OR, 1.51; β, 0.41; 95% CI, 1.01-2.25) were regarded as significant independent risk factors.

DISCUSSION

Traditionally, GERD is defined based on three major diagnostic parameters: (1) ambulatory 24-h esophageal pH monitoring; (2) upper gastrointestinal (GI) tract endoscopic examination for erosive esophagitis; and (3) clinical evaluation by physicians and clinical therapeutic treatment by acid suppression agents. The sensitivity of endoscopic examination is limited, as most patients with GERD do not have obvious mucosa injury. Therefore, most of their disease is categorized as non-erosive reflux disease (NERD). Ambulatory 24-h esophageal pH monitoring also has problems with sensitivity for the intermittent nature of symptoms and daily activities may disturb the placement of a pH probe. Endoscopy can be more easily applied to healthy participants than ambulatory 24-h esophageal pH monitoring. Furthermore, it is more objective in terms of finding reflux disease, which has been investigated in many previous studies. Erosive esophagitis is classified using the LA system, which appears to be the most unambiguous and simple method to apply. However, endoscopic examination alone can not rule out GERD or acid-induced epithelial injury. A variety of questionnaires designed for GERD clinical trials have been developed. The Gastrointestinal Symptom Rating Scale (GSRS)[10] comprises 15 items addressing five symptom clusters (gastroesophageal reflux, abdominal pain, indigestion, diarrhoea, and constipation). The GSRS used graded response categories from "none" to "very severe" without defining what these adjectives meant. This can produce subjective answers, reducing reliability and validity[11]. The "CarlssonDent Self-Administered Questionnaire (QUEST)"[12] had a good face validity, since it incorporated "word pictures" using simple English to describe symptoms of GERD. The GERQ[13] is a self-administered validated instrument that identifies the onset of GERD symptoms and grades the frequency and severity of symptoms over a prior year. It was a long questionnaire containing 80 questions, making it inconvenient for use in clinical trials.

The Chinese version of the Reflux Diagnostic Questionnaire (RDQ): Its framework of the RDQ was based on a validated questionnaire published before[3]. Shaw *et al*[3] found that the RDQ demonstrated validity and reliability and was responsive to change for reflux. The reliability coefficient of the RDQ scales ranged from 0.8 to 0.88, well beyond the acceptable level of 0.70. It was tested in the multicenters and found that it could accurately identify the presence of symptoms suggestive of GERD[9]. It was designed to measure GERD symptoms over the previous month, not the previous year. It was feasible to prevent the recall bias since McColl found that 1-mo was the maximum period over which patients could provide reliable data due to recall errors[14]. Four symptoms were included in the RDQ that may be somewhat different from the definition of the previous studies[10,12-13]. It would be more accurate to include substernal chest pain and food regurgitation to make a diagnosis of GERD[9,15]. Complete satisfaction of multitrait scaling criteria justifies combining the items into scales that can be scored with simple addition, thus eliminating the need for item weighting[16]. As our study confirmed there was no significant statistical difference between the two investigations. The Kappa-value was 0.4-0.75, which demonstrated no predominant consistency between the two diagnostic methods.

GERD becomes more common in Asian countries, resulting in more people coming to the gastroenterology outpatient department for treatment. A 13%-15% prevalence of reflux symptoms has been reported in

Asian GERD patients, which is comparable with results in many Western series. However, the definition of GERD may alter estimated prevalence. In the present study, data for subjects with GERD symptoms (23.70%) support previous prevalence rates[17].

We showed for the first time that the prevalence of NERD in the Chinese is 10.60% (237 of 2231 investigated persons), which is lower than that of erosive esophagitis (20.80%), while the prevalence rate of symptomatic GERD is 10%-30% in Western countries, and more than half of the patients lack endoscopically proven erosive esophagitis[18,19]. In Western countries, the majority of patients with GERD have been reported to have NERD, but not erosive esophagitis, even in cases with severe symptoms[18].

The factors that determine the form of NERD versus erosive esophagitis have not yet been clarified. However, we elucidated differences in the possible causative factors of NERD and erosive esophagitis. By multivariate logistic regression analysis, it was found that old age, male, moderate working burden, divorced and strong tea remained as significant independent risk factors for erosive esophagitis, meanwhile, greasy food consumption, constipation were regarded as significant independent risk factors for NERD.

Several risk factors associated with GERD have been reported as follows. Old age has been shown to be associated with increased risk of erosive esophagitis, Berrat's esophagus, and esophageal adenocarcinoma[20].

The previous studies showed that male gender is a risk factor for erosive esophagitis; whereas female is more likely to be associated with NERD[21,22]. Less parietal cell mass in women may be underlining reasons for the lower risk of GE[23].

Tea drinking has previously only been studied in a case series of reflux episodes[24]. While from another previous population-based study, the tea drinking does not seem to be a risk factor for GERD[25].

Coffee has been reported to be a reduced risk of reflux symptoms among coffee drinkers compared with non-coffee drinkers[25,26]. But previous cross sectional epidemiological studies have been able to establish that coffee drinking is a risk factor for GERD[24]. To accurately evaluate the long term effects of coffee drinking on the risk of reflux, an analysis of prospective exposure data would be necessary.

Smoking has often been cited as risk factors for GERD, although the findings of studies on this matter have been inconsistent[25,27]. Smoking was inversely related to NERD compared with RE[22]. Smoking decreases lower esophageal sphincter pressure and increases the frequency of reflux episodes. In addition, deleterious effects on esophageal defenses such as reduction of esophageal clearance and salivary function have been described[22].

Erosive esophagitis was positively related to alcohol consumption[28]. The mechanism is that alcohol intake induces nausea and vomiting and directly causes mucosal impairment, while food intake at late night elevates the risk of esophagitis[29].

In this cross sectional study, greasy food consumption was associated with an increased risk of GERD symptoms and erosive esophagitis. Several physiological studies of human volunteers have shown increased frequency of transient lower esophageal sphincter relaxation and increased esophageal acid exposure with greasy food consumption[30].

However, a limitation of the current study involves the subject sample. Subjects were outpatients from 10 hospitals and therefore probably represent a population of intermediate GERD severity between subjects recruited from gastrointestinal clinics and those randomly selected from the general population. In addition, other potential risk factors of GERD such as *H pylori*, hiatal hernia and BMI were not assessed in our study. Whether these factors are positively associated with GERD, calls for further observations.

In conclusion, GERD is a highly prevalent disease. Old age, male, moderate working burden, divorced and strong tea remained as significant independent risk factors for erosive esophagitis, meanwhile, midst bodily form, greasy food consumption, and constipation were considered as significant independent risk factors for NERD.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a common disorder with a high occurrence of up to 10%-38% of adults in the Western population at least once a week. The prevalence of GERD has been increasing year after year. The diagnosis and treatment of GERD are important because the disease, in addition to the highly disturbing typical symptoms, has a series of known consequences. The presence of GERD may affect the patients' quality of life, decrease functional activity, increase the economic burden associated and highlight the risk of esophageal carcinoma in the cases of Barrett's esophagus. With an emphasis on morphological diagnosis, endoscopy has become a major tool to assess the final consequences of GERD, which is especially useful for population-based screening.

Research frontiers

The definition or the diagnostic parameters of GERD and the factors that determine the form of NERD versus erosive esophagitis have not yet been clarified.

Innovations and breakthroughs

Although many investigators have reported the prevalence of erosive esophagitis, the prevalence of NERD has not been investigated in China. We showed for the first time that the prevalence of NERD in the Chinese is 10.60% (237 of 2231 investigated persons), which is lower than that of erosive esophagitis (20.80%), While the rate of symptomatic GERD is 10% to 30% in Western countries, and more than half of these patients lack endoscopically proven erosive esophagitis. The factors that determine the form of NERD versus erosive esophagitis have not yet been clarified. However, we elucidated differences in the possible causative factors of NERD and erosive esophagitis.

Applications

Our study was designed to analyze a spectrum of GERD subjects based on presenting symptoms and endoscopic findings. In order to determine the risk factors for such disease in outpatients from department of internal medicine in Zhejiang Province of East China, a cross-sectional survey in a cluster random sample was conducted from November 2004 to June 2005.

Peer review

This is a report designed to analyze a spectrum of GERD subjects based on presenting symptoms and endoscopic findings in Eastern part of China, surveyed by RDQ Chinese version. This clinical study was well designed.The limitation of the study was absence of assessment of the other important risk factors of GERD such as *H pylori*, hiatal hernia, and BMI. It is well known that negative association between *H pylori* and GORD does exist, especially in Asia (Kupcinskas

L, Malfertheiner P. Helicobacter. 2005; 10 Suppl 1: 26-33). In case the other GERD risk factors would be assessed, the results of multivariate analysis could be substantially different.

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RAPID COMMUNICATION

Breath test for differential diagnosis between small intestinal bacterial overgrowth and irritable bowel disease: An observation on non-absorbable antibiotics

I Esposito, A de Leone, G Di Gregorio, S Giaquinto, L de Magistris, A Ferrieri, G Riegler

I Esposito, A de Leone, G Di Gregorio, S Giaquinto, L de Magistris, A Ferrieri, G Riegler, Department of Clinical and Experimental Internal Medicine, Second University of Naples, Italy
Correspondence to: Dr. Gabriele Riegler, Department of Clinical and Experimental Internal Medicine, Second University of Naples, Italy. gabriele.riegler@unina2.it
Telephone: +39-81-5665292 Fax: +39-81-5665112
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Abstract

AIM: To estimate the prevalence of small intestine bacterial overgrowth (SIBO) among patients with an earlier diagnosis of irritable bowel disease (IBS) in our geographical area, and to collect information on the use of locally acting non-absorbable antibiotics in the management of SIBO.

METHODS: A non-interventional study was conducted in 73 consecutive patients with a symptom-based diagnosis.

RESULTS: When the patients underwent a "breath test", 33 (45.2%) showed the presence of a SIBO. After treatment with rifaximin 1200 mg/d for seven days in 32 patients, 19 (59.4%) showed a negative "breath test" one week later as well as a significant reduction of symptoms, thus confirming the relationship between SIBO and many of the symptoms claimed by patients. In the other 13 patients, "breath test" remained positive, and a further cycle of treatment with ciprofloxacin 500 mg/d was given for 7 additional days, resulting in a negative "breath test" in one patient only.

CONCLUSION: (1) about half of the patients with a symptomatic diagnosis of IBS have actually SIBO, which is responsible for most of the symptoms attributed to IBS; (2) only a "breath test" with lactulose (or with glucose in subjects with an intolerance to lactose) can provide a differential diagnosis between IBS and SIBO, with almost identical symptoms; and (3) the use of non-absorbable antibiotics may be useful to reduce the degree of SIBO and related symptoms; it must be accompanied, however, by the correction of the wrong alimentary habits underlying SIBO.

Key words: Small intestine bacterial overgrowth; Irritable bowel disease; Breath test; Rifaximin; Hydrogen; Non-absorbable antibiotics

Esposito I, de Leone A, Di Gregorio G, Giaquinto S, de Magistris L, Ferrieri A, Riegler G. Breath test for differential diagnosis between small intestinal bacterial overgrowth and irritable bowel disease: An observation on non-absorbable antibiotics. *World J Gastroenterol* 2007; 13(45): 6016-6021

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INTRODUCTION

Irritable bowel syndrome (IBS) is a very common diagnosis in gastroenterology that is done on the basis of the Rome Ⅱ symptomatic criteria. The basic clinical pattern is characterized by abdominal pain and changes in bowel habit, on the basis of which three different variants of IBS are recognized (IBS with stipsis, IBS with diarrhea or IBS with alternated stipsis and diarrhea). No matter which variant is diagnosed, 92% of the patients with IBS complain of abdominal bloating, flatulence and meteorism, three symptoms that are, however, more probably related to a small intestine bacterial overgrowth (SIBO) rather than to IBS.

A close relationship exists between the changes in pattern and distribution of gastrointestinal (GI) bacterial flora, and the altered GI motility (changes in bowel habit) and sensorial physiology (abdominal pain and bloating) observed in patients with IBS. It has been demonstrated that the myoelectric activity of intestinal loops are deeply modified by the presence of SIBO, leading to the hypothesis that many of the sensorial and motorial symptoms of IBS are really determined by changes in the GI bacterial flora[1]. Moreover, it is well known that both an acute GI infection[2,3] and the use of systemic antibiotics[4,5] lead to profound changes in GI bacterial flora, and that both the conditions may result in symptoms (such as abdominal bloating and changes in bowel habit), which look like those of IBS[6-9].

Finally, it has been reported that even one single cycle of systemic antibiotics may provoke long-time sustained alterations of GI physiology[10], while a treatment with antibiotics specifically addressed to correction of intestinal

disbiosis is followed by an improvement of IBS- or SIBO-related symptoms[11]. Thus, there is ground to believe that there is a large overlapping between SIBO and IBS, and that many patients with an earlier symptomatic diagnosis of IBS are actually suffering from SIBO. However, the prevalence of SIBO among patients with an initial diagnosis of IBS is not exactly known.

Cuoco and Salvagnini[12] have recently reported in North Italy a 46% incidence of positive "breath test" (increased hydrogen concentrations in the expired air after oral lactulose administration) among 96 patients with IBS. According to USA-based clinicians, this incidence could be higher than 80%[13-15], while European investigators have reported an increased GI bacterial flora in 43% of patients with IBS compared with 12% of matched-control healthy subjects, without any relationship between degree of disbiosis and severity of altered GI motility and symptoms[16].

The different values in SIBO prevalence observed worldwide among patients with an initial diagnosis of IBS are probably due to the different methods employed to detect the bacterial colonization of the small intestine: a typical and simple clinico-laboratory test ("breath test" with lactulose) in the first two studies[14,15], a more rigorous microbiological, but also methodologically more complicated test (GI bacterial count $\geqslant 10^5$/mL) in the third study[16].

Recent studies have provided increasing support for the concept that disturbances in gut flora occur in patients with IBS and that such abnormalities may contribute to IBS-type symptoms[17]. In any case, the overlapping of SIBO and IBS and the role eventually played by SIBO in the pathogenesis of the IBS symptoms, are demonstrated by two double-blind placebo-controlled clinical studies, which have shown respectively a 75% reduction in the GI symptoms and a long-lasting (over 10 wk) clinical improvement in subjects with IBS, after treatment with non-absorbable antibiotics with a topical activity limited to the GI tract[18].

Our study is therefore aimed to estimate the prevalence of SIBO in our geographical area (Campania, South Italy) in patients with IBS diagnosed according to the Rome Ⅱ criteria; the diagnosis of SIBO is established on the basis of a positive "breath test" with lactulose. We have also gathered information on the use of locally active antibiotics in the management of SIBO.

MATERIALS AND METHODS

This study was purely observational. Within a time interval of 27 mo (January 2005-March 2007), we selected patients of both sexes who came to our medical centre for advice, and had a diagnosis of IBS, because of abdominal pain and discomfort complying with the following characteristics: (1) Three months of continuous or recurring symptoms of abdominal pain or irritation that: (a) may be relieved with a bowel movement; (b) may be coupled with a change in frequency, or (c) may be related to a change in the consistency of stools. (2) Two or more of the following present at least 25% of time: (a) change in stool frequency (> 3 bowel movements daily or < 3 bowel movements weekly); (b) noticeable difference in stool form (hard, loose and watery stools or poorly formed stools); (c) passage of mucous in stools; (d) bloating or feeling of abdominal distention; (e) altered stool passage (e.g. sensations of incomplete evacuation, straining, or urgency).

Patients with severe cardiovascular or respiratory or renal diseases and patients with cancer or under treatment with antibiotics and corticosteroids were excluded. All the patients gave their informed consent to the management of personal data according to the "privacy" regulations.

All the symptoms, either GI or not, were recorded during the first medical visit, and the patients were asked to score the global intensity of symptoms by means of Visual Analogue Scale (VAS) 10-cm long (0 = no symptom; 10 = unbearable symptom). Then, all the patients underwent a "breath test", whose concept is based on a non-invasive measurement of hydrogen (H_2) concentrations in the expired air.

In the evening before the examination, the patient was required to eat only boiled rise with no sausage or cheese, and grilled meat, to make a careful oral hygiene and to drink only no-gas water. If stipsis was present, the dietary prescriptions were extended to the three days preceding the exam. On the day of the test, the patient was completely fasted, and smoking was forbidden. Immediately before the test two samples of expired air were taken at a 10-min interval to assay the basal hydrogen concentrations in the still fasted subject; then, 75 g of lactulose were administered and the expired air was sampled every 15 min in the next 3 consecutive hours. In one subject with intolerance to lactose, the "breath test" has been performed by using 50 g of glucose and sampling expired air every 10 min for 2 h.

A positive test required an elevated breath hydrogen concentration higher than 10 ppm over basal values[19]; these concentrations are indicative of a bacterial colonization of the small intestine, where bacteria can metabolize non-absorbable sugars thus producing increased H_2 amounts which are eliminated through respiration[20].

The patients with a positive "breath test", were diagnosed as having SIBO and treated with rifaximin polimorph A (Normix®, Alfa Wassermann) at the daily dose of 1200 mg/die for 7 consecutive days. One week after the end of the treatment, the "breath test" was repeated, and the patients who still showed a positive test, received a further treatment with ciprofloxacin 500 mg/die for additional 7 d. At the end of the second cycle of antibiotic treatment the "breath test" was repeated for the third time.

The demographic characteristics of the patients were described as means and standard deviations (min-max ranges), or frequencies when appropriate. The frequencies of symptoms observed in patients with diagnosis of SIBO and IBS were compared using the χ^2 test, and the frequency of positive "breath test" was analyzed by means of the Fisher exact test.

RESULTS

A summary flow-chart of the employed methodology and the results achieved in our study is shown in Figure 1.

A total of 73 patients with IBS were selected (28 males and 45 females). They were aged between 17 and 87 (mean ± SD, 41.2 ± 15.8 years), and their weight and height were 66.8 ± 12.6 kg and 167.1 ± 9.3 cm,

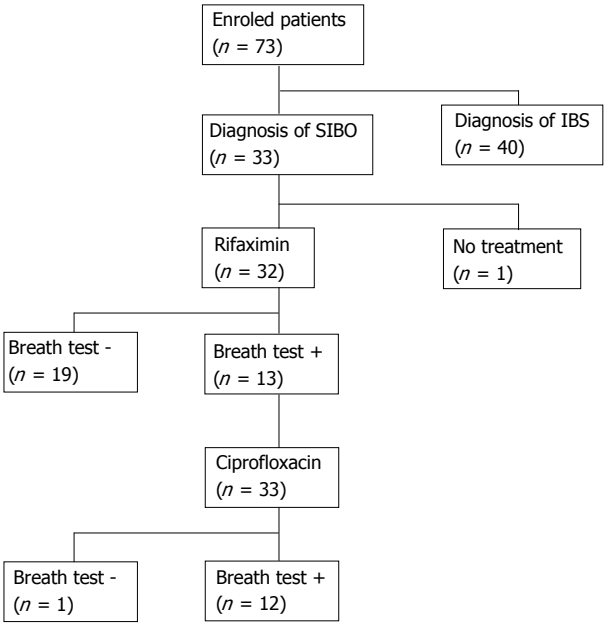

Figure 1 Diagram and synthesis of activities and results in this study.

Table 1 Results of "breath test" with lactulose or glucose in 73 patients with an initial symptoms-based diagnosis of IBS

Definitive diagnosis, *n* (%)		Breath test	Lactulose	Glucose
SIBO	33 (45.2%)	Positive	32	1
IBS	40 (54.8%)	Negative	40	0

The data are reported as frequency and percentage. SIBO: Small intestine bac-terial overgrowth; IBS: Inflammatory bowel disease.

respectively. More than 60% of males and 50% of females were younger than 40, and 10% of both males and females were older than 60 years.

The symptoms more frequently observed were abdominal bloating (83.6%), lower abdominal pain (76.7%), flatulence (65.8%), tenesmus (63.0%) and pain to palpation (50.7%), followed with lower frequencies by chronic diarrhoea, upper abdomen pain, nausea, steatorrhea, reduced body weight and stipsis. It is interesting to note that the most frequently observed symptom ("abdominal bloating") is also the most characteristic symptom of SIBO.

When the patients underwent the "breath test" with lactulose (except one patient with intolerance to lactose who received a "breath test" with glucose), 33 (45.2%) had a positive test, revealing the presence of a clinically relevant bacterial contamination of the small intestine (Table 1).

The symptoms in the patients with a confirmed diagnosis of IBS and those with a diagnosis of SIBO (positive "breath test") are shown in Table 2. The symptomatology was almost superimposable in the two groups, although some symptoms, such as reduced body weight, nausea, pain to palpation, and chronic diarrhoea, were slightly less frequent in subjects with SIBO, while other symptoms, such as tenesmus, were slightly more frequent in the patients with IBS. On the whole, the analysis of the clinical symptoms confirmed that a "breath test" is needed for a differential diagnosis between SIBO and IBS.

Table 2 Frequency of symptoms in 40 and 33 patients with a definitive diagnosis of IBS and SIBO respectively

	IBS (*n* = 40) *n* (%)	SIBO (*n* = 33) *n* (%)	*P*
Chronic diarrhoea	16 (40.0)	17 (51.5)	NS
Upper abdominal pain	17 (42.5)	14 (42.4)	NS
Lower abdominal pain	30 (75.0)	26 (78.8)	NS
Tenesmus	28 (70.0)	18 (54.5)	NS
Pain to palpation	18 (45.0)	19 (57.6)	NS
Abdominal bloating	32 (80.0)	29 (87.9)	NS
Flatulence	24 (60.0)	24 (72.7)	NS
Reduced body weight	7 (17.5)	9 (27.3)	NS
Nausea	9 (22.5)	15 (45.4)	NS
Steatorrhea	3 (7.5)	1	NS
Megaloblastic anemia	1	-	NS
Stipsis	8 (20.0)	9 (27.3)	NS
Fever	1	2 (6.1)	NS
Other (not specified)	1	0	NS

The data are reported as frequency and percentage.

Table 3 Results of "breath test" and symptom score in 32 patients with defini-tive diagnosis of SIBO treated with rifaximin 1200 mg/d for 7 d

	Before treatment	After treatment with rifaximin	
Breath test	Positive	Positive	Negative
	32 (100.0%)	13 (40.6%)	19 (59.4%)
Global symptom score	3.48 ± 0.82	3.24 ± 0.80	0.91 ± 0.06
		NS	*P* = 0.004
		After a further antibiotic treatment with ciprofloxacin	
Breath test		Positive	Negative
		12	1
Global symptom score		3.32 ± 0.95	1.00

The test was repeated one week later and, in the subjects with a still positive "breath test", a further treatment with ciprofloxacin was done (the data are re-ported as frequencies or mean ± standard deviation as appropriate).

Except one patient who refused further treatment, all the patients showing a positive "breath test" were treated with rifaximin 1200 mg/d for seven days. Among them, 19 (59.4%) patients showed the disappearance of the hydrogen peaks in expired air at the "breath test" one week after the treatment. In these patients, the symptom score was significantly reduced from 3.48 ± 0.82 (basal) to 0.91 ± 0.06 after treatment with rifaximin ($P = 0.004$), thus confirming the relationship between SIBO and many of the symptoms claimed by patients (Table 3).

On the contrary, the remaining 13 subjects still showed a positive "breath test" in spite of a treatment with rifaximin, and reported a symptom score (3.24 ± 0.80) that was almost unchanged compared with the basal values. In these patients, a further antibiotic treatment was given with ciprofloxacin 500 mg/d for 7 additional days. At the end of the treatment, only one patient showed a negative "breath test", while in the remaining 12 patients the "breath test" was still positive and the symptom score remained unchanged (Table 3).

No adverse effect or adverse drug reaction was observed in our study during the test and/or the medicinal treatment.

DISCUSSION

The GI tract is colonized by bacteria immediately after birth[21]; *Escherichia coli*, *Streptococci* and *Clostridi* are the first bacteria harboured by the colon, followed by anaerobic *Enterococci*, *Lattobacilli* and *Bacteroidi*[22]. All these bacteria are able to bind the GI mucosa by means of receptors, such as adhesin and lectin, which are expressed either on the host mucosa or other bacteria[23,24], and to resist to the antibacterial activity of many substances that are present in the GI environment, as well as to the gastric acid and GI motility[25].

Many factors affect the type and distribution of the bacteria along the GI tract, starting from the type of delivery[26] and nursing[27] in the first days of life, up to the food habits during adulthood. Normally, bacteria are scarcely present in the acid environment of the stomach while they reach the highest concentrations in the large intestine[28]. Moreover, the pattern of bacterial colonization is different among the different segments of the GI tract, the most prevalent being bacteria represented by aerobes and gram-positive in the duodenum and proximal ileum[28], gram-negative in the distal ileum, and anaerobes (*Bacteroides*, *Bifidobacteri*, *Eubacteri* and *Clostridii*) in the colon[28-30].

The role played by the bacterial flora in the normal physiology of the GI tract is known from animal studies performed many years ago[31-34]. It is quite clear nowadays that the bacterial flora affects the GI motility by means of three different mechanisms: (a) the release of substances produced or metabolized by bacteria; (b) the involvement of neuroendocrine factors; and (c) the involvement of the GI immunological tissue.

The growth of bacteria is controlled by several mechanisms, including gastric acid secretion, immunological factors, diet and bacterial competition[28,29,35]; however, the GI motility is probably the most important factor for control of bacterial growth. It is known that a large part of the bacteria may be eliminated by drugs increasing the GI motility. More importantly, a reduced GI motility leads to bacterial colonization of the small intestine, and many systemic and/or GI diseases characterized by a reduced GI motility have SIBO as one of their consequences[36,37].

In our study, slightly lower than 50% of patients with an initial diagnosis of IBS, are actually affected by a SIBO that is responsible for many of the symptoms earlier attributed to IBS. Our estimate is almost identical to the 46% observed by other clinicians in North Italy with similar methods of investigation[12].

Although other investigators have found a lower prevalence using the direct complex method of the GI bacterial count, it should be noted that the "breath test" with lactulose or glucose, with the determination of the hydrogen concentrations in the expired air, is considered an indirect but highly specific, method for diagnosis of SIBO[38,39]. On the other hand, the symptomatology in both SIBO and IBS is almost identical (Table 2) and, therefore, only a "breath test" can help in the differential diagnosis between the two disorders.

Clinicians should be encouraged to perform a "breath test" to promptly identify a SIBO, because the disorder has several systemic consequences ranging from malabsorption of lipids and liposoluble vitamins and loss of electrolytes[22,28], to a more severe translocation of bacteria (usually, gram-negative and aerobic bacteria, such as *Escherichia*, *Proteus*, *Enterobacter* and *Klebsiella*) from the GI tract to extraintestinal tissues[40], especially in the presence of a pathologically reduced epithelial barrier and immunological defences[41,42]. All these factors may lead to sepsis and multiorgan failure[42-46].

The treatment of SIBO must firstly focus on the correction of wrong food and dietary habits that usually underlie the disorder (e.g. excessive use of fast-food), and then to the reduction of bacterial colonization of the small intestine by means of antibiotics[47,48]. In this regard, the use of locally acting non-absorbable antibiotics would be particularly useful in reducing immediately the bacterial count waiting for the slow-acting beneficial effects of dietary measures.

In our study, the treatment with rifaximin for one week has determined the negativization of "breath test" in 59.4% of treated patients. Our data confirm other reports in the most recent literature: at the dose of 800 mg for four weeks rifaximin significantly reduced the symptoms in 20 patients with IBS and led to a negative "breath test" in almost half of patients[49]; in another series of 23 patients with SIBO and positive "breath test", a treatment with rifaximin 1200 mg/d for 7 d followed by a treatment with probiotics, led to a negative "breath test" in 19 (82.6%) cases and significantly reduced the peak in hydrogen concentrations in the expired air from 40.9 ± 20.4 to 4.78 ± 8.42 ppm[12]. More evidence on the efficacy of rifaximin has been reported in patients with SIBO and acute diverticulitis of the colon[50], and patients with SIBO and celiac disease[51].

It should be noted that further treatment with ciprofloxacin - an antibiotic widely used in the treatment of IBS[52,53] has not given significantly better results than rifaximin in our experience. Valuable alternatives to rifaximin that have been proven to be effective in the treatment of SIBO are represented by norfloxacin and amoxicillin-clavulanic acid[54], gentamycin[55], trimethoprim/sulfamerazin and polymyxin[56], and chlortetracycline[57].

In conclusion, (1) about half of the subjects with a symptomatic diagnosis of IBS have SIBO as a main cause of their claimed symptoms, which have been initially imputed to IBS; (2) only a "breath test" either with lactulose or with glucose in subjects with intolerance to lactose, can provide a differential diagnosis between IBS and SIBO with identical symptoms; (3) the use of non-absorbable antibiotics is useful in reducing the degree of GI bacterial contamination and related symptomatology, although the correction of wrong dietary habit remains the milestone in the management of SIBO if we want to maintain the results achieved with antibiotic treatment for quite some time.

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COMMENTS

Background

The role played by the bacterial flora in the normal physiology of gastrointestinal

(GI) tract is known, and it is quite clear nowadays that the bacterial flora affects the GI motility by means of three different mechanisms: (a) the release of substances produced or metabolized by bacteria; (b) the involvement of neuroendocrine factors; and (c) the involvement of the GI immunological tissues. Recent studies have provided increasing support for the concept that disturbances in gut flora occur in patients with irritable bowel syndrome (IBS) and such abnormalities may contribute to IBS-type symptoms.

Research frontiers

The article provides evidences that in about 50% of patients with a symptom-based diagnosis of IBS, the symptoms are provoked by a small intestine bacterial overgrowth (SIBO).

Innovations and breakthroughs

Clinicians should be encouraged to perform a "breath test" to promptly identify a SIBO, because the disorder has several systemic consequences of malabsorption of lipids and liposoluble vitamins, and loss of electrolytes.

Applications

The treatment of SIBO must firstly focus on the correction of wrong food and dietary habits that usually underlie the disorder (e.g. excessive use of fast-food), and then to the reduction of bacterial colonization of small intestine by means of antibiotics. In this regard, the use of locally acting non-absorbable antibiotics would be particularly useful in reducing immediately the bacterial count waiting for the slow-acting beneficial effects of dietary measures.

Terminology

A standard microbiological definition of SIBO: an increased bacterial count in the small intestine $\geqslant 10^5$ colonic bacteria/mL). A positive "breath test": an elevated breath hydrogen concentration within 90 min, two distinct peaks, and an increase higher than 20 ppm over basal values; these concentrations are indicative of a bacterial colonization of the small intestine, where bacteria can metabolize non-absorbable sugars, thus increasing the H_2 amounts which are eliminated through respiration.

Peer review

This report documents the incidence of small bowel overgrowth in patients with irritable bowel syndrome and how their symptoms respond to appropriate antibiotic therapy and whether or not the overgrowth (documented by serial hydrogen breath tests) is eradicated.

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RAPID COMMUNICATION

Predictive factors of tumor response to trans-catheter treatment in cirrhotic patients with hepatocellular carcinoma: A multivariate analysis of pre-treatment findings

Roberto Miraglia, Giada Pietrosi, Luigi Maruzzelli, Ioannis Petridis, Settimo Caruso, Gianluca Marrone, Giuseppe Mamone, Giovanni Vizzini, Angelo Luca, Bruno Gridelli

Roberto Miraglia, Luigi Maruzzelli, Settimo Caruso, Gianluca Marrone, Giuseppe Mamone, Angelo Luca, Department of Radiology, Mediterranean Institute for Transplantation and Advanced Specialized Therapies (IsMeTT), University of Pittsburgh Medical Center, Palermo 90127, Italy
Giada Pietrosi, Ioannis Petridis, Giovanni Vizzini, Department of Hepatology, Mediterranean Institute for Transplantation and Advanced Specialized Therapies (IsMeTT), University of Pittsburgh Medical Center, Palermo 90127, Italy
Bruno Gridelli, Department of Transplantation Surgery, Mediterranean Institute for Transplantation and Advanced Specialized Therapies (IsMeTT), University of Pittsburgh Medical Center, Palermo 90127, Italy
Correspondence to: Roberto Miraglia, Department of Radiology, Mediterranean Institute for Transplantation and Advanced Specialized Therapies (IsMeTT), University of Pittsburgh Medical Center, IsMeTT, Via Tricomi 1, Palermo 90127, Italy. rmiraglia@ismett.edu
Telephone: +39-91-2192111 Fax: +39-91-2192344
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Abstract

AIM: To elucidate the pre-treatment clinical and imaging findings affecting the tumor response to the transcatheter treatment of unresectable hepatocellular carcinoma (HCC).

METHODS: Two hundred cirrhotic patients with HCC received a total of 425 transcatheter treatments. The tumor response was evaluated by helical CT and a massive necrosis (MN) was defined as a necrosis > 90%. Twenty-five clinical and imaging variables were analyzed: uninodular/multinodular HCC, unilobar/bilobar, tumor capsula, hypervascular lesion, portal vein thrombosis, portal hypertension, ascites, platelets count, aspartate transaminases/alanine transaminases (AST/ALT), alfa-fetoprotein (AFP) > 100, AFP > 400, serum creatinine, virus hepatitis C (VHC) cirrhosis, performance status, age, Okuda stage, Child-Pugg stage, sex, CLIP (Cancer of the Liver Italian Program) score, serum bilirubin, constitutional syndrome, serum albumine, prothrombin activity, BCLC (Barcelona Clinic Liver Cancer) stage. Prognostic factors of response were subjected to univariate analysis and thereafter, when significant, to the multivariate analyses.

RESULTS: On imaging analysis, complete response was obtained in 60 (30%) patients, necrosis > 90% in 38 (19%) patients, necrosis > 50% in 44 (22%) patients, and necrosis < 50% in 58 (29%) patients. Ninety-eight (49%) of the 200 patients were considered to have a MN. In univariate analysis, significant variables (P < 0.01) were: uninodular tumor, unilobar, tumor size 2-6 cm, CLIP score < 2, absence of constitutional syndrome, and BCLC stage < 2. In a multivariate analysis, the variables reaching statistical significance were: presence of tumor capsule (P < 0.0001), tumor size 2-6 cm (P < 0.03), CLIP score < 2 (P < 0.006), and absence of constitutional syndrome (P < 0.03). Kaplan-Mayer cumulative survival at 12 mo was 80% at 24 mo was 56%. MN was associated with a longer survival (P < 0.0001).

CONCLUSION: MN after transcatheter treatment is more common in the presence of tumor capsule, maximum diameter of the main lesion between 2 and 6 cm, CLIP score < 2 and absence of constitutional syndrome. The ability to predict which patients will respond to transcatheter treatment may be useful in the clinical decision-making process, and in stratifying the randomization of patients in clinical trials.

Key words: Hepatocellular carcinoma; Trans-catheter embolization/chemoembolization; Tumor response

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INTRODUCTION

Trans-catheter treatment is extensively used to treat hepatocellular carcinoma (HCC) not suitable for surgical resection or percutaneous ablation therapies. Transarterial chemoembolization (TACE), transarterial oily chemoembolization (TOCE) and transarterial embolization

(TAE) have been adopted[1-8]. Prognosis of patients with HCC complicated with cirrhosis mainly depends on the tumor growth, progression of the underlying liver disease, and effectiveness of anti-tumor treatment. Recent meta-analysis showed that the overall survival of patients with well preserved liver function was improved after intra-arterial treatment[9,10]. The primary goal of the trans-catheter treatment is to achieve a massive necrosis, to reduce tumor size, and prevent its dissemination and portal vein invasion. Unfortunately, the tumor response to trans-catheter treatment is heterogeneous with a wide range of necrosis that cannot be accurately predicted. The lack of well-defined prognostic indicators inspired us to perform the present study to analyze the tumor response and the pre-treatment imaging and clinical prognostic factors predictive of response in patients with HCC and compensated cirrhosis who were treated by trans-catheter treatment.

Table 1 Patient and HCC characteristics at admission

Age (mean ± SD, range)	63 ± 8.62 (35-81)
Sex distribution (male/female)	138/62
Liver cirrhosis etiology:	
Hepatitis C	158
Hepatitis B	29
Others	13
Child Pugh score A/B/C	136/59/5
Clip score (0/1/2/3/4/5)	53/79/52/11/4/1
BCLC stage (1/2/3/4)	61/115/14/0
Constitutional syndrome (yes/no)	22/178
Performance status (0/1/2/3)	177/14/8/1
HCC uninodular/multinodular	96/104
HCC unilobar/bilobar	148/52
Tumor capsula: yes/no	83/114
Portal vein thrombosis: absent/partial/complete	185/15/0
Alfa-fetoprotein: < 20/20-100/> 100 ng/mL	83/48/69

MATERIALS AND METHODS

This is a retrospective cohort study based on the analysis of 200 consecutive cirrhotic patients with single or multifocal HCC, treated with intra-arterial therapy and evaluated with follow-up imaging at a single transplant centre. Patients who had at least one image examination (16-slides helical CT scan or triphasic contrast-enhanced MRI) before and after treatment were included into the study. Diagnosis of HCC was based on radiological findings, alfa-fetoprotein level and biopsy according to the Barcelona criteria[11]. Bone metastases were ruled out by bone scintigraphy; lung and abdominal metastases were ruled out by CT scan. Liver function impairment was estimated with routine biochemical parameters reflecting liver function. The cardiac risk was evaluated by EKG and left ventricular ejection fraction (LVEF), as measured by multi-gated angiography scan (MUGA scan) or echocardiography. HCC and patient's characteristics at admission are reported in Table 1. Informed consent was not specifically required for the study, although written informed consent was obtained for each diagnostic and interventional radiology procedure.

All CT scan studies were performed with a 16-slice multidetector CT (Light speed, General Electric Medical Systems, USA) before and 4-6 wk after the intra-arterial treatment. Quadruple-phases protocol was used (unenhanced phase, arterial phase, portal venous phase and late phase).

All CT images were evaluated by at least 2 radiologists. Pre-treatment studies were analyzed without knowledge of the final outcomes of the patients. A consensus of the readers was reached in all cases. The following CT features were evaluated: number of lesions (single, multiple), size of the main lesion (maximum diameter), hepatic distribution (unilobar or bilobar), tumor extension ($\leqslant$ 3 lesions each $\leqslant$ 3 cm or > 3 lesions or one > 3 cm), vascularity of the lesions (hypervascular, hypovascular), tumor capsule, portal vein invasion (lobar, segmental, or subsegmental), signs of portal hypertension (spontaneous splenorenal shunt, patency of umbelical vein, patency of coronary vein), ascites, and necrosis. In post-treatment studies, the presence of arterial enhancement at CT imaging was considered as viable tumor. In patients who underwent TOCE, the complete necrosis was considered only if the lesion had homogeneous Lipiodol uptake without contrast enhancement in arterial phase. In case without clear results, MRI with gadobenate dimeglumine (Gd-BOPTA), using a 1.5T MR scan (General Electric Medical Systems, USA), was performed. The efficacy of intra-arterial treatment was defined according to the amount of tumor necrosis valuable on CT and/or MR follow-up imaging and the WHO recommendations[12]. The presence of non-enhanced tumor areas was defined as tissue necrosis and expressed as percent of the total tumor volume. Tumor necrosis was considered complete when no foci of enhancement were seen within the tumor or at its periphery. In patients with multiple lesions, the necrosis was computed as average of tumor necrosis in each lesion. The response to treatment was classified as: complete response, necrosis > 90%, necrosis > 50%, and necrosis < 50%. In this study, a tumor necrosis > 90% over the treatment interval was classified as massive necrosis (MN).

Intra-arterial treatment was performed in patients with multifocal HCC or single unresectable HCC and contraindications to radiofrequency thermal ablation (RFA).

In our centre, contraindications to RFA are: size of the lesion greater than 4 cm, lesion near to vital organs such as gallbladder, stomach, and colon, lesion adjacent to a big portal or hepatic vein branches at risk of bleeding, subphrenic lesion not easily accessible for RFA and lesion in subcapsular position at high risk of tumor seeding[13].

Exclusion criteria for intra-arterial treatment were HCC volume > 50% of total hepatic volume, complete thrombosis of main portal vein, AST/ALT > 300 U/L, serum bilirubin > 3 mg/dL, serum creatinine > 1.8 mg/dL, white blood cell (WBC) < $2.5 \times 10^3/\mu L$, platelets < $35 \times 10^3/\mu L$, severe ascites and performance status > 3.

TACE was performed using Epirubicine at a dose of 50 mg/m^2 of body surface; the dose was reduced to 50% if serum bilirubin level was > 1.2 mg/dL and < 2 mg/dL and/or white blood cell count (WBC) was $34 \times 10^3/\mu L$;

a dose reduction to 25% was administered if bilirubin was > 2 mg/dL and/or WBC 2.53 × 10^3/μL. Epirubicine was prepared in sterile drip and infused over 30 min using a peristaltic pump. Afterwards, the embolization was performed using Gelfoam (Pfitzer, Belgium) till a stagnation flow was visualized at the fluoroscopy. In patients with acceptable liver function and superselective catherization of the hepatic artery, 2-10 mL of Lipiodol (Lipiodol Ultrafluid, Guebert, Italy) was infused before the gelfoam embolization (TOCE)[14]. No chemotherapeutic agent (TAE) was used in the presence of WBC less than 2.5 × 10^3/μL, previous episodes of neutropenia (< 500/mL), positive HBsAg and HBV DNA[15] and ejection fraction ⩽ 45%. These patients were treated with lipiodol and/or gelfoam. In patients with single HCC, superselective catheterization of the artery supplying the lesion was performed whenever possible, in the other cases the treatment was given in the branch of the right hepatic artery or in the branch of the left hepatic artery supplying the lesion. In patients with multifocal HCC, treatments were given in the right hepatic artery (RHA) or in the left hepatic artery (LHA). In case of multifocal, bilobar HCC, no treatment was given in RHA and LHA during the same session. After the treatment, the patients were carefully observed in a conventional hospitalization room, and analgesics were administered if necessary. Oral intake was reinitiated as soon as possible according to the tolerance of the patients. Usually the day after the procedure, after confirming the absence of clinical abnormalities, the patients were discharged and followed up in the outpatient clinic.

The intra-arterial treatment was repeated every 6-12 wk according to the tumor response based on the follow-up imaging and clinical assessment.

Statistical analysis

Twenty-five clinical and imaging variables were analyzed, including uninodular/multinodular HCC, unilobar/bilobar, tumor capsula, hypervascular lesion, portal vein thrombosis, portal hypertension, ascites, platelets count, AST/ALT, AFP > 100, AFP > 400, serum creatinine, VHC cirrhosis, performance status, age, Okuda stage, Child-Pugg stage, sex, CLIP score, serum bilirubin, constitutional syndrome, serum albumine, prothrombin activity, BCLC stage.

The relationship between these variables and the tumor response was first assessed by univariate analysis using the Chi-square test or Student's *t* test when indicated. For qualitative variables, patients were grouped according to the presence or absence of each variable. For quantitative variables, the cut-off level was determined evaluating the relationship between the sensitivity and the specificity of the MN at different cut-off points from a ROC curve. To identify independent predictors of MN occurrence, all the variables reaching statistical significance in the univariate analysis were subsequently included in a multivariate analysis using the step-wise logistic regression procedure. A *P* value of less than 0.05 was considered significant. Statistical analyses were performed with the SPSS software (SPSS Institute Inc., Cary, NC). Survival curves were modelled using the Kaplan-Meier method.

RESULTS

Patients received a total of 425 sessions of intra-arterial treatments. The mean number of treatment sessions was 2.1 ± 2.0 per patient, range 1-8. Interval of treatment was 76 ± 48 d (range 16-249 d). Type of treatments performed were: TACE 243 (57%), TOCE 126 (30%) and TAE 56 (13%). Technical success was achieved in all the treatments performed. No major life-threatening complications occurred.

On imaging analysis, complete response was obtained in 60 (30%) patients, necrosis > 90% in 38 (19%) patients, necrosis > 50% in 44 (22%) patients, and necrosis < 50% in 58 (29%) patients. In this analysis, 98 (49%) of the 200 patients were considered to have MN. At univariate analysis, significant variables ($P < 0.01$) were: uninodular tumor, unilobar, tumor size 2-6 cm, CLIP score < 2, absence of constitutional syndrome, and BCLC stage < 2. In a multivariate analysis, the variables reaching statistical significance were: presence of tumor capsule (β-coefficient 1.447, $P < 0.0001$), tumor size 2-6 cm (β-coefficient 0.838, $P < 0.03$), CLIP score < 2 (β-coefficient 1.074, $P < 0.006$), and absence of constitutional syndrome (β-coefficient 1.764, $P < 0.03$). Kaplan-Mayer cumulative survival was 80% at 12 mo and 56% at 24 mo. The survival of patients with and without MN was 95% and 75% at 12 mo, and 70% and 55% at 24 mo, respectively. Massive tumor necrosis was associated with a longer survival ($P < 0.0001$).

DISCUSSION

Transcatheter treatment is the most common choice for patients with surgically unresectable HCC and contraindications to percutaneous treatment such as PEI, and RF thermal ablation. The arterial embolization with or without chemotherapy induces tumor necrosis by occlusion of the feeding artery of the HCC, and its clinical efficacy has been documented[3,4,16-21]. The goal of TAE/TACE is to deliver a high dose of chemotherapeutic drug and/or embolizing agent in the HCC, causing tumor necrosis and tumor control, preserving as much normal liver parenchyma as possible. The imaging and clinical factors affecting the tumor response after transcatheter treatment remain to be elucidated. The aim of this study was to identify, by means of multivariate analyses, the pre-treatment variables of independent predictive value of MN in patients with cirrhosis managed with transcatheter therapy.

Our multivariate analysis showed that two tumor-related factors such as tumor capsule and maximum diameter of the main tumor are the only independent factors significantly affecting the tumor response to the transcatheter treatment. The presence of a well-recognizable tumor capsule at the pretreatment CT scan was the most important independent predictive factor of MN after transcatheter treatment. According to our results, previous pathological studies performed on liver resection following transarterial chemoembolization[22,23] demonstrated that encapsulated HCCs are more responsive to the transcatheter treatment than non-encapsulated tumors. Interestingly, a significant correlation

between the thickness of the capsule and the effectiveness of tumor necrosis after chemoembolization has also been documented[24,25]. The reason for the relatively poor necrosis of tumors without capsule is not clear. Wasaka *et al* showed that barium sulfate infused into the portal vein entered into the non-encapsulated tumors but not into encapsulated tumors, suggesting that there is a difference in the type of blood supply that may greatly influence the tumor necrosis after embolization of the feeding hepatic artery[23]. The type of histologic growth pattern at the tumor-nontumor boundary may also affect the necrosis occurrence after transcatheter treatment. HCC is usually the expanding type in encapsulated tumors and replacing type in uncapsulated tumors and chemoembolization appears to be most efficacious for HCC with growth expanding pattern. The poor results expected in the replacing type of HCC may be related to the fact that in the case of tumor, cells replace hepatocytes, the blood spaces communicate with sinusoids supplied by portal blood flow[26,27]. A limitation of the pathological studies is that they were performed weeks or months after the chemoembolization, and the granulated tissue between HCC and normal liver parenchyma, secondary to the treament response, could simulate a true capsule. In this case, the psuedocapsule could be an indicator and not a predictor of tumor response[28]. Our results showed that the naturally formed capsule of encapsulated HCC resulting from condensed reticulin fibers produced by atrophic changes of noncancerous liver tissue and detected at the pretreatment CT scan, is the most important independent predictive factor to obtain MN after transcatheter treatment. However, the capsule seems to form when the tumors are small, and larger tumors are commonly uncapsulated. Finally, in absence of a very well-defined capsule, one must keep in mind the possibility that a negative CT image may not completely exclude the presence of capsule and may give partial information about its integrity. It is known the CT scan is limited in detecting capsular invasion of tumor cells, small satellite nodules, and tumor thrombi of the peripheral portal vein branches that are rather poorly responsive to the transcatheter treatment probably because they receive blood supply from the portal vein[23-25,28].

We obtained MN after transcatheter treatment more frequently in nodules with a maximum diameter of 2-6 cm. Similarly, a previous histologic study evaluating the effect of chemoembolization showed that it is effective for encapsulated HCC measuring between 2.3 and 5.5 cm in diameter[25]. TAE is usually less effective for HCC smaller than 2 cm in diameter[27,29]. The reason for the failure of a complete necrosis of the small tumors is not clear. No significant difference of the predominant lesion vascular pattern was found in this study between patients with or without MN. The apparent discrepancy from previously reported studies[30] might be due to the fact that in our series the hypovascular pattern was very uncommon.

A CLIP score < 2 and the absence of constitutional syndrome are strong independent clinical predictors of MN after transcatheter treatment because these kind of patients have a preserved liver function and multiple treatments might yield a good response.

In conclusion, pretreatment helical CT and clinical findings provide accurate prediction of tumor response in patients with HCC and compensated cirrhosis. The MN (> 90%) is more common in the presence of capsule, maximum diameter of 2-6 cm and in well compensated patients with CLIP score of 1 and without constitutional syndrome. The ability to predict which patients will respond to transcatheter treatment may be useful in the clinical decision-making process, and in stratifying the randomization of patients in the therapeutic clinical trials. The prognostic implications and the survival of patients with massive tumor necrosis require further studies.

COMMENTS

Background

Trans-catheter treatment is extensively used to treat hepatocellular carcinoma in cirrhotic patients to achieve a massive necrosis, to reduce tumor size, and prevent its dissemination and portal vein invasion. Tumor response after trans-catheter treatment cannot be accurately predicted.

Research frontiers

The aim of this study is to analyze tumor response and the pre-treatment imaging and clinical prognostic factors predictive of response in patients with HCC and compensated cirrhosis who were managed by trans-catheter treatment.

Innovations and breakthroughs

This study clearly showed that massive necrosis is more common in presence of capsule, a maximum diameter of 2-6 cm and in well compensated patients with CLIP score of 1 and without constitutional syndrome.

Applications

The ability to predict which patients will response to transcatheter treatment may be useful in the clinical decision-making process and in stratifying the randomization of patients in clinical trials.

Terminology

CLIP (Cancer of the Liver Italian Program) prognostic score includes Child-Pugh stage, tumor morphology and extension, serum alfa-fetoprotein (AFP) levels, and portal vein thrombosis. Costitutional syndrome is characterized by weight loss, malaise and anorexia.

Peer review

This is an interesting study, aiming at identifying the factors predicting tumor response to the trans-catheter treatment in cirrhotic patients with HCC. The authors concluded that presence of tumor capsule, a maximum diameter between 2 and 6 cm, CLIP score < 2 and absence of constitutional syndrome were independent predictors of massive necrosis. The study is well conducted. The results are clearly reported and support the main conclusions.

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RAPID COMMUNICATION

Evaluation of leukocyte esterase and nitrite strip tests to detect spontaneous bacterial peritonitis in cirrhotic patients

Serkan Torun, Enver Dolar, Yusuf Yilmaz, Murat Keskin, Murat Kiyici, Melda Sinirtas, Emre Sarandol, Selim Gurel, Selim Giray Nak, Macit Gulten

Serkan Torun, Yusuf Yilmaz, Uludag University Medical School, Department of Internal Medicine, Bursa 16059, Turkey
Enver Dolar, Murat Keskin, Murat Kiyici, Selim Gurel, Selim Giray Nak, Macit Gulten, Uludag University Medical School, Department of Gastroenterology, Bursa 16059, Turkey
Melda Sinirtas, Uludag University Medical School, Department of Microbiology and Infectious Diseases, Bursa 16059, Turkey
Emre Sarandol, Uludag University Medical School, Department of Biochemistry, Bursa 16059, Turkey
Correspondence to: Yusuf Yilmaz, MD, Uludag Universitesi Tip Fakultesi Ic Hastaliklari, Gorukle, Bursa 16059, Turkey. yusufyilmaz@uludag.edu.tr
Telephone: +90-533-4403995 Fax: +90-224-4534028
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Abstract

AIM: To investigate the diagnostic efficacy of leukocyte esterase and nitrite reagent strips for bedside diagnosis of spontaneous bacterial peritonitis (SBP).

METHODS: A total of 63 consecutive patients with cirrhotic ascites (38 male, 25 female) tested between April 2005 and July 2006 were included in the study. Bedside reagent strip testing was performed on ascitic fluid and the results compared to manual cell counting and ascitic fluid culture. SBP was defined as having a polymorphonuclear ascites count of $\geqslant 250/mm^3$.

RESULTS: Fifteen samples showed SBP. The sensitivity, specificity, positive and negative predictive values of the leukocyte esterase reagent strips were; 93%, 100%, 100%, and 98%, respectively. The sensitivity, specificity, positive and negative predictive value of the nitrite reagent strips were 13%, 93%, 40%, and 77%, respectively. The combination of leukocyte esterase and nitrite reagents strips did not yield statistically significant effects on diagnostic accuracy.

CONCLUSION: Leukocyte esterase reagent strips may provide a rapid, bedside diagnostic test for SBP.

Key words: Spontaneous bacterial peritonitis; Urinary reagent strip; Leukocyte esterase; Nitrite

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INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a serious and potentially life-threatening complication that can occur in cirrhotic patients with ascites[1,2]. The prevalence of SBP in hospitalized patients with liver cirrhosis and ascites is high, ranging between 10% and 30%. Notably, in-hospital mortality rates range between 30% and 50%[3,4].

SBP requires timely diagnosis that is usually based on cytobacteriological examination of ascitic fluid. A polymorphonuclear leucocyte (PMN) cell count in ascitic fluid > $250/mm^3$, irrespective of the ascitic fluid culture, is currently considered the standard criterion for diagnosis of SBP[1,2]. For this testing, it is of paramount importance that the results of the cytobacteriological examination of the ascitic fluid should be promptly delivered so that appropriate antibiotherapy can be initiated[4,5]. However, because of the organization of facilities in many hospitals, a bacteriological laboratory is not always available for all departments admitting cirrhotic patients with ascites. It follows that alternative methods for rapid diagnosis of SBP are an urgent requirement[6,7].

Use of reagent strip testing for leucocyte esterase has been proposed to reduce the time from paracentesis to a presumptive diagnosis of SBP from a few hours to a few seconds[7,8]. Intriguingly, reagent strips detecting leukocyte esterase activity in biological fluids have been validated for the diagnosis of urinary tract infections[9,10], peritonitis in patients on continuous ambulatory peritoneal dialysis[11], pleural infections[12] and meningitis[13]. The observation that nitrate levels in ascites fluid are raised in patients with SBP is also of interest[14]. Thus far, however, no study has specifically looked at the potential usefulness of nitrite reagent test strips as a diagnostic tool in this patient group.

Therefore, in this study we sought to determine the sensitivity, specificity, and positive (PPV) and negative predictive values (NPV) of nitrite and leukocyte esterase reagent test strips for the diagnosis of SBP in cirrhotic patients with ascites.

MATERIALS AND METHODS

Study sample

This hospital-based study was conducted at the Department of Gastroenterology, Uludag University Medical School, Bursa, Turkey from April 2005 to July 2006. Sixty-three consecutive unselected cirrhotic patients (38 men, 25 women, mean age 59.0 ± 11.6 years) with ascites were included in our study. The general characteristics of the study participants are shown in Table 1. Diagnosis of cirrhosis was established by histological criteria and/or by clinical, laboratory, endoscopic and/or ultrasonographic findings. Only subjects with serum-ascites albumin gradient (SAAG) > 1.1 g/dL were enrolled. Patients with SAAG < 1.1 g/dL were excluded. Subjects with portal hypertension (SAAG > 1.1 g/dL) due to malignancy or tuberculosis were also excluded. The local ethics committee of the Uludag University Medical School approved the study and informed consent was obtained from each participant.

Paracentesis

Paracentesis was performed at admission and during the hospital stay for treatment of ascites or with clinical signs of SBP. Immediately after the paracentesis, ascitic fluid was tested using nitrite and leukocyte esterase reagent strips (Aution Sticks 10 EA; Arkray, Kyoto, Japan). Fresh ascitic fluid was collected in a clean dry container and the strip was immediately immersed in ascitic fluid. The strips were read by two independent research physicians who were unaware of the clinical status of the participants. The strips had a colorimetric 5-grade scale (0-4). A correlation between PMN and a 5-grade scale was suggested by the manufacturer, as follows: grade 0, 0 PMN/mm^3; grade 1, 25 PMN/mm^3; grade 2, 75 PMN/mm^3; grade 3, 250 PMN/mm^3; and grade 4, 500 PMN/mm^3.

Laboratory analysis of the ascitic fluid was performed without delay in all patients. For testing a standard sterile technique was used and included total and differential cell counts, Gram stain and total protein levels. Cultures of ascitic fluid were performed at bedside for all patients using blood culture bottles, including both aerobic and anaerobic media. A minimum of 10 mL of ascitic fluid was inoculated into each bottle.

Diagnostic criteria

The diagnosis of SBP was based on a PMN cell count > 250/mm^3 in ascitic fluid, irrespective of a positive ascitic fluid culture and clinical signs of SBP, and an absence of intra-abdominal sources of infection, inflammation or tuberculosis.

Statistical analysis

Sensitivity was defined as the proportion of patients with a positive reagent strip divided by the number of those with SBP diagnosed by criteria previously defined. Specificity was defined as the proportion of patients with a negative reagent strip divided by the total number of patients without SBP. PPV was defined as the proportion of patients with a true-positive reagent strip divided by the total number of patients with a positive reagent strip. NPV was defined as the proportion of true-negative reagent strips divided by the total number of patients with a negative reagent strip. Data are presented as means ± SD for quantitative variables and as frequencies for qualitative variables. The exact 95% confidence interval for each statistic was calculated from the binominal distribution. SPSS statistical software version 14.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis.

Table 1 General characteristics of the study participants

Characteristics	Study patients (n = 63)
Male gender, n (%)	38 (60.3)
Age (yr)	59 ± 11.6
Child–Pugh classification, n (%)	
B	25 (39.7)
C	38 (60.3)
Etiology of cirrhosis, n (%)	
Chronic B hepatitis	19 (30.1)
Chronic C hepatitis	11 (17.5)
Alcohol abuse	7 (11.1)
Other causes	26 (41.3)

RESULTS

According to the Child-Pugh classification, of the 63 patients there were 25 (39.7%) with Child class B and 38 (60.3%) with Child class C. The etiology of cirrhosis was chronic B hepatitis in 19 (30.1%), chronic C hepatitis in 11 (17.5%), alcohol abuse in 7 (11.1%), and other causes (e.g., autoimmune, primary biliary cirrhosis, cryptogenic) in 26 (41.3%).

SBP occurred in 15 patients (23.8%). Culture of ascitic fluid was positive in 6 cases (40.0%) and negative in 9 (60.0%). Two patients (3.0%) were found to have infected ascites fluid, whereas the remaining 46 (73.0%) had no sign of ascites infection. The bacteria isolated from cultures of ascitic fluid were: *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus warneri*, and *Streptococcus bovis*. PMN counts in ascitic fluid in patients with or without SBP were (mean ± SD/mm^3) 4064 ± 4486 and 69 ± 63, respectively.

Among the 15 patients with SBP, the urine leukocyte esterase strip tested 1+ in the ascitic fluid of one patient and ⩾ 2+ in the remaining 14 individuals. The ascitic fluids of 2 patients with SBP tested positive by the nitrate strips. Detailed laboratory data and urine strip test results of the 15 SBP patients are shown in Table 2. The sensitivity, specificity, PPV and NPV of the leukocyte esterase reagent strips (at a ⩾ 2+ cutoff point) were as follows: 93%, 100%, 100%, and 98%, respectively. The sensitivity, specificity, PPV and NPV of the nitrite reagent strips were 13%, 93%, 40%, and 77%, respectively. The addition of nitrite strips to leukocyte esterase reagent strips did not yield statistically significant effects upon the diagnostic accuracy compared to the leukocyte esterase test alone. Thus the combination of both tests yielded a sensitivity, specificity, PPV and NPV of 93%, 100%, 100%, and 98%, respectively.

DISCUSSION

SBP continues to be an important source of morbidity and mortality in patients with cirrhosis[15]. Under such

circumstances, prompt diagnosis and treatment are crucial to ensure better clinical outcomes in this patient group. However, it has been shown that a positive bacterial culture can be obtained in just 40% of patients with SBP, and that test results may be delayed for several days[16]. Under such circumstances, reagent strips emerge as an attractive means for rapid diagnosis of this clinical entity in patients with cirrhosis and ascites. These tests have the ability to detect esterase activity of PMN cells[16]. Numerous independent studies have evaluated the diagnostic value of reagent strips in settings involving SBP[17-19]. Most have shown high sensitivity and specificity in keeping with our present results.

In a landmark study by Castellote *et al*[8], it has been shown that reagent strips are useful and inexpensive when used in the context of SBP. Specifically, when a cutoff point of 2 or more was selected, overall sensitivity and specificity were 96% and 98%, respectively[6]. More recently, Thévenot *et al*[18] have investigated the utility of two reagent strips, the Multistix test and the Combur 2 test, for the rapid diagnosis of SBP. Both tests showed a high sensitivity and specificity and permitted an accurate laboratory diagnosis of this life-threatening condition. Furthermore, a multicenter study by Sapey *et al*[19] has provided important evidence that two leukocyte esterase reagent strips (Nephur-Test and MultistixSG10) may be of clinical use in the bedside diagnosis of SBP. In light of these results, it has been suggested that in patients with cirrhosis and positive strip test results, antibiotic therapy should be started without delay[17]. Our study confirms and expands previous findings of the potential utility of the leukocyte esterase test as a simple and inexpensive means for testing in settings involving SBP[18-20]. Notably, the selection of a cutoff point $\geqslant 2$ yielded 100% specificity and 100% PPV for the diagnosis of SBP. By contrast, results of a urine strip test of 0 and 1+ yielded a 98% NPV, which excluded SBP in these individuals.

Another aim of our study was to determine whether nitrite reagent strip testing is a useful diagnostic tool in patients with SBP. Previous reports have indicated that nitrate concentration and nitric oxide levels may be raised in ascites from patients with cirrhosis and SBP[14,21]. Since nitric oxide is a diffusible, short-lived, and a reactive free radical gas that is difficult to measure *in vivo*[22,23], most studies have based their conclusions on measurement of nitrite and nitrate in biological fluids[24-26]. In line with this approach, in our study we used Aution Sticks 10EA for the nitrite test strips. This test is based on the Griess Reaction which measures the combined oxidation products for nitric oxide (nitrites and nitrates) after reduction with nitrate reductase[24]. Nonetheless, the sensitivity and PPV of the nitrite reagent strips were low, namely 13% and 40%, respectively. There are several reasons that might explain the low diagnostic performances of the nitrite reagents strips. Nitrite and nitrate levels in body fluids may vary according to a host of confounding factors including the effects of diet-derived nitrate, hepatic synthesis capacity, as well as the occurrence and prevalence of concomitant infections[27-29]. Specifically, it is posited that the limited utility of nitrite strips to screen for SBP in patients with cirrhosis may be due to the low bacterial concentration in ascites fluid[30]. Therefore, for precise bedside diagnosis of SBP in patients with cirrhotic ascites, the highly specific leukocyte esterase strip test remains the method of choice.

Table 2 Results of urine strip test, culture, and ascitic fluid PMN count in 15 patients with SBP

Case number	Reagent strip test		Culture	PMN count (/mm^3)
	Leukocyte esterase	Nitrite		
1	1+	0	Negative	490
2	4+	0	*Escherichia coli*	3860
3	2+	0	Negative	510
4	3+	0	Negative	802
5	3+	0	Negative	311
6	4+	0	*Escherichia coli*	7130
7	4+	1+	*Klebsiella pneumoniae*	16700
8	2+	0	*Staphylococcus warneri*	280
9	4+	0	*Streptococus bovis*	3810
10	3+	0	Negative	9100
11	3+	0	Negative	6120
12	3+	0	Negative	2460
13	4+	0	Negative	5330
14	3+	0	Negative	3790
15	2+	2+	*Klebsiella pneumoniae* plus *Escherichia coli*	270

In conclusion, leukocyte esterase reagent test strips were found to facilitate very rapid identification of patients with SBP and cirrhotic ascites. Specifically, this test can be performed efficiently in order to speed up the bedside diagnostics of this clinical entity. It fits easily into the work flow of a routine gastroenterology department and can be conducted in facilities that do not have the facilities to carry out biochemical and bacteriological tests for diagnosis of SBP.

COMMENTS

Background

SBP is a serious and potentially life-threatening complication that can occur in patients with cirrhosis and ascites. Timely diagnosis is of paramount clinical importance.

Research frontiers

Use of reagent strip testing for leucocyte esterase has been proposed to reduce the time from paracentesis to a presumptive diagnosis of SBP from a few hours to a few seconds.

Innovations and breakthroughs

The leukocyte esterase reagent test strips were found to enable the very rapid identification of patients with SBP in clinical settings involving patients with cirrhotic ascites. This test can be performed efficiently to speed up bedside diagnostics of this clinical entity.

Applications

Leukocyte esterase reagent strip testing fits easily into the work flow of a routine gastroenterology department and can be conducted in facilities that do not or cannot carry out biochemical and bacteriological tests for diagnosis of SBP.

Terminology

Reagent-strip leukocyte esterase: the presence of leukocyte esterase is indirect evidence of the presence of white blood cells in biological fluids.

Peer review

The manuscript "Evaluation of leukocyte esterase and nitrite strip tests to detect spontaneous ascites infection in cirrhotic patiens" is very interesting since it evaluates a test for prompt diagnosis of a disastrous complication of cirrhosis with poor prognosis. In general terms, the study was well performed and the analysis was good.

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RAPID COMMUNICATION

Alterations in the function of circulating mononuclear cells derived from patients with Crohn's disease treated with mastic

Andriana C Kaliora, Maria G Stathopoulou, John K Triantafillidis, George VZ Dedoussis, Nikolaos K Andrikopoulos

Andriana C Kaliora, Maria G Stathopoulou, George VZ Dedoussis, Nikolaos K Andrikopoulos, Department of Science of Dietetics-Nutrition, Harokopio University, Athens, Greece
John K Triantafillidis, Department of Gastroenterology, Saint Panteleimon General State Hospital, Nicea, Athens, Greece
Supported by a grant from the Chios Gum Mastic Growers Association
Correspondence to: Dr. Andriana C Kaliora, Department of Science of Dietetics-Nutrition, Harokopio University of Athens, 70 El. Venizelou ave., Kallithea 17671, Athens, Greece. akaliora@hua.gr
Telephone: +30-210-9549303
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Abstract

AIM: To assess the effects of mastic administration on cytokine production of circulating mononuclear cells of patients with active Crohn's disease (CD).

METHODS: The study was conducted in patients with established mildly to moderately active CD, attending the outpatient clinics of the hospital, and in healthy controls. Recruited to a 4 wk treatment with mastic caps (6 caps/d, 0.37 g/cap) were 10 patients and 8 controls, all of who successfully completed the protocol. Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), macrophage migration inhibitory factor (MIF) and intracellular antioxidant glutathione (GSH) were evaluated in peripheral blood mononuclear cells (PBMC) before and after treatment.

RESULTS: Treating CD patients with mastic resulted in the reduction of TNF-α secretion (2.1 ± 0.9 ng/mL *vs* 0.5 ± 0.4 ng/mL, $P = 0.028$). MIF release was significantly increased (1.2 ± 0.4 ng/mL *vs* 2.5 ± 0.7 ng/mL, $P = 0.026$) meaning that random migration and chemotaxis of monocytes/macrophages was inhibited. No significant changes were observed in IL-6, MCP-1 and GSH concentrations.

CONCLUSION: This study shows that mastic acts as an immunomodulator on PBMC, acting as a TNF-α inhibitor and a MIF stimulator. Although further double-blind, placebo-controlled studies in a large number of patients is required to clarify the role of this natural product, this finding provides strong evidence that mastic might be an important regulator of immunity in CD.

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory condition of unknown cause[1]. Although the exact pathogenesis of CD is poorly understood, infection, environmental factors, heredity and immunological defects have been proposed as causes[2]. In one or another scenario, a variety of cytokines, such as tumor necrosis factor-alpha (TNF-α), are secreted at the site of inflammation by intestinal lamina propria and attract and activate effector cells[3-5]. Apart from the intestinal mucosa, TNF-α concentration is also raised in serum in patients with active Crohn's disease, compared to normal controls[6,7] and inactive disease[8]. The mechanism through which localized inflammation in the gastrointestinal tract in active CD is associated with systemic manifestations remains unconfirmed, but can involve activated mononuclear cells that migrate *via* the peripheral blood (PBMC) to other tissues. PBMC are highly activated in active CD and secret higher quantities of proinflammatory mediators[9-11]. Altered production of cytokines by PBMC is noted in patients with CD when compared to healthy subjects[12].

As the name implies, monocyte chemotactic protein 1 (MCP-1) acts as a potent chemoattractant and activator of monocytes/macrophages[13], as well as of NK cells, T cells, eosinophils, and basophils[14-17]. During acute inflammatory processes the expression of MCP-1 is increased. Once activated, cells produce an assortment of immunoregulatory cytokines that influence the course of the ensuing immune response. Interleukin 6 (IL-6) expressed among others by T cells and monocytes/

macrophages, stimulates T- and B-cell proliferation and differentiation[18]. The production of IL-6 in these various cells may be regulated by TNF. TNF-α is an important mediator of inflammation, found in substantial amount in the mucosa and stools of subjects with CD. It is a proinflammatory cytokine produced by monocytes, macrophages, and T cells that can affect proliferation, differentiation, and function of every cell type. Therefore, TNF inhibitors, which may be useful in the treatment of CD, have recently been developed[19]. Among these, infliximab has been developed as a therapeutic agent for TNF-α -mediated diseases[20]. Macrophage migration inhibitory factor (MIF) is a cytokine with dissimilar functions; inhibition of macrophage migration[21] or proinflammatory activity[22-25].

Nowadays, various immunosuppressive therapies such as azathioprine, mercaptopurine, and methotrexate, are available. Nevertheless, the treatment of patients with CD still remains a clinical challenge. Furthermore, in view of the increased number of CD patients, there is a considerable scientific and commercial interest in the discovery of novel classes of therapeutic compounds. In particular, plants represent a good source of novel molecules. *Pistacia lentiscus* var. *Chia* (*Anacardiaceae*) is an evergreen shrub widely distributed in the Mediterranean region. Mastic, the resinous exudate, has been reported to possess antioxidant[26] and antibacterial[27] activity, to be effective against peptic ulcers[28], to be hepatoprotective in tetrachloride-intoxicated rats[29] and to suppress the extent of iron-induced lipid peroxidation in rat liver homogenates[30]. We have previously shown that mastic administration resulted in the improvement of the clinical course and in the regulation of inflammatory biomarkers in plasma obtained from mildly to moderate active CD patients[7]. The aim of the present work was to investigate the immunomodulatory effect of mastic treatment on cytokine secretion. Additionally, because inflammation results in oxidative stress and endogenous antioxidants, such as glutathione (GSH), do not counteract it with subsequent mucosal damage, intracellular GSH production from PBMC obtained from patients with mildly to moderately active CD was also measured.

MATERIALS AND METHODS

Setting and participants

Ten consecutive patients with active Crohn's disease and eight healthy controls were included[7]. In brief, clinical evidence of mild to moderate Crohn's disease exacerbation was defined by a score of CD Activity Index (CDAI) 150 < CDAI < 400. Mean CDAI at baseline was 222.9 ± 18.7 (SE), while mean C-reactive protein (CRP) concentration was 40.3 ± 13.1 (SE) mg/mL. Exclusion criteria were elemental diet, parenteral nutrition or antioxidant/mineral supplementation and treatment with immunomodulators (biologic agents-infliximab) and/or corticosteroids. Controls were healthy volunteers, with normal concentrations of CRP [2.4 ± 0.7 (SE) mg/L] and albumin [42.1 ± 1.2 (SE) g/L], without chronic inflammatory disorder, BMI value < 30 (25.8 ± 3.3), none anti-inflammatory drug treatment or antioxidant vitamin/mineral supplementation. Informed consent was obtained from each subject included in the study. The Ethical Committees of both Harokopio University and Saint Panteleimon General State Hospital approved the protocol. Table 1 shows some demographic characteristics of patients and controls.

Table 1 Demographic characteristics and medications of patients with Crohn's Disease and controls

Characteristic	Patients	Controls
Age (yr)		
Mean	36.9	31.5
Range	18-73	25-45
Sex		
Female	5	4
Male	5	4
Duration of disease (yr)	6.4 (± 3.9)	-
Concomitant medication		-
None	3	
Mesalazine	3	-
Metronidazole	2	
Azathioprine	2	-
Location of Crohn's disease		
Small bowel	4	-
Small and large bowel	6	-
Fistulizing disease	3	-

Intervention

The trial protocol was carried out as previously described[7]. In short, participants were subjected to a 4-wk supplementation with mastic caps (0.37 g/cap, 2 × 3 caps/d, 2.2 g in total). Dietary instructions were given as to maintain consumption of foods rich in anti-inflammatory and antioxidant ingredients low as initially assessed by a food frequency questionnaire and 24 h recall interview and to refrain from mastic and mastic products. Blood samples were obtained prior and after the trial.

Cell cultures

PBMC were obtained from CD patients and controls as previously described[31]. Viability of peripheral blood mononuclear cells (PBMC) was determined by Trypan blue exclusion test. PBMC were resuspended in complete medium consisting of RPMI 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin. PBMC were added to each well of a 24-well plate at a density of 2×10^6 cells/mL and cultures were incubated at 37℃ in a humidified atmosphere containing 5% CO_2 for 18 h. At the end of incubation, conditioned media were collected and stored at -20℃ until assayed, while cells were harvested for GSH measurement. All cultures were run in duplicate.

Cytokine assays

Plasma cytokines from patients with CD and controls were assessed by quantitative, sandwich, enzyme-linked, immunosorbent assays (ELISA) (R&D Systems Abingdon, UK) according to the manufacturer's instructions. Sensitivity of TNF-α ELISA was less than 1.6 pg/mL, of

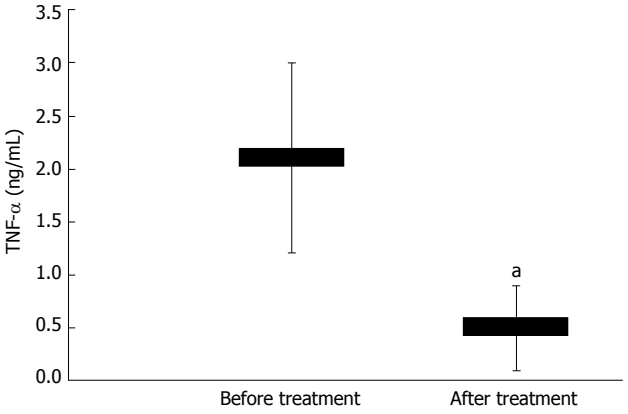

Figure 1 Secretion of tumor necrosis factor-alpha (TNF-α) was decreased in PBMC derived from patients with active Crohn's disease (n = 10) after 4-wk treatment with mastic caps ($^aP < 0.05$). Horizontal bars represent the mean ± SE.

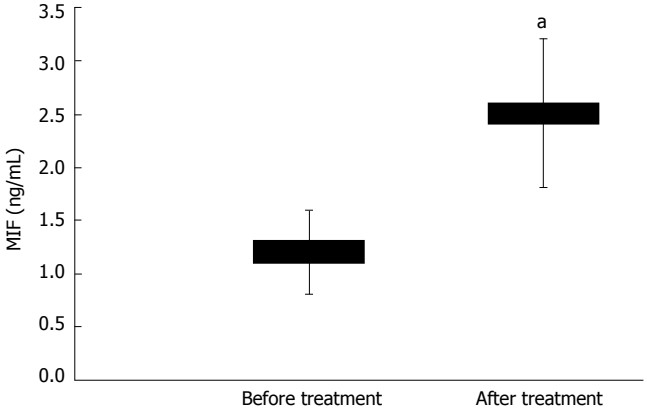

Figure 2 Secretion of macrophage migration inhibitory factor (MIF) was increased in PBMC derived from patients with active Crohn's disease (n = 10) after 4-wk treatment with mastic caps ($^aP < 0.05$). Horizontal bars represent the mean ± SE.

IL-6 was less than 0.70 pg/mL, of MIF less than 0.017 ng/mL and of MCP-1 less than 5.0 pg/mL.

Assay for GSH

At the end of incubation, cells were MPA-treated (5%) and then centrifuged at 2000 × g for 10 min. The resulting supernatant was separated and the GSH assay was performed using the Colorimetric Assay for Glutathione as indicated by the manufacturer (OxisResearch Inc., Portland, USA). GSH concentration was evaluated using a standard curve of Absorbance Units *vs* GSH concentrations and expressed as μmol/L.

Statistical analysis

Results are expressed as mean ± SE. The Mann-Whitney Test was used for comparing differences between patients and controls prior treatment. Differences reported primarily and at the end of the study within individual groups, were tested for significance by the Wilcoxon signed ranks test. A P value below 0.05 was regarded as limit of significance.

RESULTS

Secretion of TNF-α

Our data show that PBMC isolated from patients with active CD exhibited no significant difference in the production of TNF-α compared to controls prior to treatment (2.1 ± 0.9 ng/mL *vs* 1.2 ± 0.3 ng/mL, P = 0.200). TNF-α in controls administered mastic caps was not significantly changed (1.2 ± 0.3 ng/mL *vs* 0.5 ± 0.2 ng/mL, P = 0.173), while in patients it was significantly reduced (2.1 ± 0.9 ng/mL *vs* 0.5 ± 0.4 ng/mL, P = 0.028, Figure 1).

MIF production

MIF production was significantly increased in control group before mastic treatment compared to patients (4.7 ± 1.0 ng/mL *vs* 1.2 ± 0.4 ng/mL, P = 0.038). MIF production in controls administered mastic caps was not altered (4.7 ± 1.0 ng/mL *vs* 4.7 ± 0.7 ng/mL, P = 0.593), while, as shown in Figure 2, it was significantly increased in patients after mastic treatment (1.2 ± 0.4 ng/mL *vs* 2.5 ± 0.7 ng/mL, P = 0.026).

IL-6 and MCP-1

PBMC isolated from patients with active CD exhibited significantly elevated secretion of IL-6 compared to controls prior to therapy (622.5 ± 130.1 pg/mL *vs* 56.7 ± 20.2 pg/mL, P = 0.014). No significant difference was observed in controls before and after treatment (56.7 ± 20.2 pg/mL *vs* 19.9 ± 7.5 pg/mL, P = 0.285). In patients, a trend towards statistical significance was observed in IL-6, before and after treatment although, differences did not reach statistical significance (622.5 ± 130.1 pg/mL to 519.9 ± 176.5 pg/mL, P = 0.068).

In the case of MCP-1, no significant difference was observed between patients and controls prior to treatment (3.0 ± 1.1 ng/mL *vs* 0.7 ± 0.3 ng/mL, P = 0.302). MCP-1 secretion from controls' PBMC (0.7 ± 0.3 ng/mL *vs* 0.6 ± 0.3 ng/mL, P = 0.593) or from patients' PBMC (3.0 ± 1.1 ng/mL *vs* 1.7 ± 1.0 ng/mL, P = 0.463) before and after the trial was not significantly changed.

Intracellular GSH

No significant difference in intracellular GSH was detected between controls and CD patients before mastic treatment (66.4 ± 20.1 μmol/L *vs* 34.0 ± 15.8 μmol/L, P = 0.144). No significant difference was observed in GSH before and after treatment in controls (66.4 ± 20.1 μmol/L *vs* 55.4 ± 9.7 μmol/L, P = 0.285), whereas, even though not statistically significant, a trend towards statistical significance was observed in patients (34.0 ± 15.8 *vs* 56.6 ± 10.3 μmol/L, P = 0.075).

DISCUSSION

Four-week mastic administration has been previously shown to effectively regulate the clinical course, inflammation, and oxidative stress in CD patients. It statistically decreased CD activity index and plasma concentrations of C-reactive protein and IL-6, while it increased plasma total antioxidant potential. Also, nutrition risk index (NRI), one of the most useful measures of nutritional status that incorporates albumin level and body

weight, was improved[7]. In particular, the main element of NRI showing improvement was body weight gain, and since the daily energy intake was unchanged during the trial, increase in body weight and in NRI was attributed to decrease of liquid stools and consequent improvement in nutrient absorption. In two out of ten NRI was > 100 denoting adequate nutrient absorption and absence of nutritional risk. As a continuation of our research to evaluate the effect of mastic on CD and before conducting placebo-controlled studies in large cohorts, in the current study we demonstrated that mastic administration affects cytokine secretion from PBMC obtained from CD patients. Mastic acts as an immunomodulator (a) inhibiting the secretion of TNF-α and (b) inducing the secretion of MIF.

In the report of Grip and coworkers[32] TNF-α was significantly elevated in plasma obtained from CD patients, but not in PMBC, compared to healthy controls. Accordingly, the difference in TNF-α concentrations between patients and controls at baseline was significant in plasma[7], but insignificant in PBMC (present study). Interestingly though, secretion of TNF-α showed a significant decrease in CD patients subjected to mastic treatment (Figure 1). Even though the data reported about TNF-α is conflicting[33,34], the anti-TNF-α treatment in TNF-mediated diseases is developing. The mechanism of mastic's anti-TNF activity in CD may be related to specific blockade of TNF-α secretion. Because cells were always viable in all the experimental conditions, the mechanism including complement mediated lysis of cells expressing membrane bound TNF-α[35], is fairly excluded. A possible approach would be *via* the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. By blocking HMG-CoA reductase on human monocytes, cells reduce the production of TNF-α[32]. TNF-α is suggested to regulate MCP-1 secretion *via* the activation of nuclear factor-kappa B[36]. Yet, this is rather unlikely to be seen hereby, given that MCP-1 concentration was unaffected in CD patients administered with mastic. It is rather that the nuclear factor-kappa B pathway secondary to the decrease in TNF-α was not activated.

To shed more light on the mechanisms by which mastic might work, we also investigated the potency of mastic caps in affecting MIF secretion. Originally, MIF was described as an inhibitor of migration and chemotaxis of monocytes/macrophages[21]. Our findings of suppressed secretion of MIF in CD mononuclear cells compared to healthy prior treatment indicate that monocytes are sensitized to chemotaxis. Increased secretion after treatment (Figure 2) points to the inhibition of monocyte chemotaxis. The significance of this finding is that migration of chemokine or peptide or nonpeptide stimulated monocytes and differentiation to macrophages into the site of inflammation is limited and further trigger of inflammation is controlled. In some studies it is proposed to have proinflammatory properties and be the first cytokine appearing, followed by others[37]. However, since in our present and previous study[7] mastic administration was not followed by enhanced proinflammatory production, induction of MIF secretion should only be allied with inhibition of chemotaxis.

Even though insignificant, a marginal increment in intracellular GSH concentration ($P = 0.075$) was observed. GSH is the most abundant non-enzymatic antioxidant present in cells that plays an important role in the defence against oxidative-stress-induced cell injury[38]. During inflammatory processes, cells of the immune system are exposed to large amounts of reactive oxygen intermediates, and, thus, an efficient GSH system to neutralize free radicals that otherwise disturb immune functions is essential[39]. Mastic has been proven to induce GSH production in PBMC under oxidative conditions *in vitro*[26], while in CD patients to increase plasma total antioxidant potential *in vivo*[7].

IL-6 is thought to play a crucial role in the pathogenesis of CD[40]. We hereby demonstrated that IL-6 secretion in PBMC from CD patients was significantly elevated compared to healthy controls ($P = 0.014$), evident of the cytokine role in CD inflammation. While in plasma IL-6 was statistically decreased with mastic administration[7], in PBMC insignificant nevertheless decrease ($P = 0.068$) was reported, perhaps due to the small number of samples.

Cytokines play a central role in the modulation of the immune system and they have either proinflammatory, such as TNF-α, or antiinflammatory functions. In CD patients the imbalance between proinflammatory and antiinflammatory cytokines brings about the rationale for "anticytokine" treatment. It is however uncertain whether only one cytokine should be targeted or several pro- and antiinflammatory cytokines, or cytokine synthesis inhibitors, soluble receptors, receptor antagonists or receptor antibodies. In the case of mastic, the activity in CD shown previously[7] and hereby may well be extremely interesting. However, further studies -now in progress- are needed as to determine the target and whether there is one or a class or more than one class of compounds acting synergistically to obtain this effect. As a final point, mastic might serve well in the regulation of immunity in CD patients.

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COMMENTS

Background

Mastic administration improves the clinical course and regulates plasma inflammatory and antioxidative mediators of patients with mildly to moderately active Crohn's disease (CD). We aimed to assess the effects of mastic administration on cytokine production of circulating mononuclear cells of patients with active CD.

Research frontiers

The exact pathogenesis of Crohn's disease (CD) is poorly understood; infection, environmental factors, heredity and immunological defects have been proposed as causes. Peripheral blood mononuclear cells (PBMC) are highly activated in active CD and secret higher quantities of proinflammatory mediators. In view of the increased number of CD patients and of the severe side effects of the immunosuppressive therapies available there is a considerable scientific and commercial interest in the discovery of novel classes of therapeutic compounds.

Innovations and breakthroughs

This is the very first study regarding the effect of mastic administration on cytokine production of circulating mononuclear cells of patients with active Crohn's disease.

Applications

Although further double-blind, placebo-controlled studies in a large number of patients is required to clarify the role of this natural product, this finding provides strong evidence that mastic might be an important regulator of immunity in Crohn's disease.

Peer review

This study does provide some new information about a novel possible treatment for Crohn's disease.

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RAPID COMMUNICATION

Hepatitis B viral infection in maintenance hemodialysis patients: A three year follow-up

Ya-Li Cao, Shi-Xiang Wang, Zuo-Min Zhu

Ya-Li Cao, Shi-Xiang Wang, Department of Blood Purification, Beijing Chaoyang Hospital, Affiliate of Capital Medical University, Beijing 100020, China
Zuo-Min Zhu, Department of Clinical Laboratory, Beijing Chaoyang Hospital, Affiliate of Capital Medical University, Beijing 100020, China
Correspondence to: Shi-Xiang Wang, Department of Blood Purification, Beijing Chaoyang Hospital, Affiliate of Capital Medical University, Beijing 100020, China. tzg1219@hotmail.com
Telephone: +86-10-85231455
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Abstract

AIM: To observe the prevalence of hepatitis B virus (HBV) infection in maintenance hemodialysis patients.

METHODS: Eighty-eight hemodialysis patients who had been receiving hemodialysis regularly for an average of 39.45 ± 7.57 (range from 36 to 49) mo were enrolled in this study. HBV markers were measured in these patients before hemodialysis and in 100 healthy controls by the chemiluminescent microparticle immunoassay (CMI) method in order to compare the incidence of HBV infection in hemodialysis patients versus normal healthy people. All patients were then divided into two groups: patients positive for HBV markers (i.e. those positive for HBsAg, anti-HBc, HBeAg, anti-HBe, with or without positive anti-HBs) (n = 33), and patients negative for HBV markers (including those only positive anti-HBs) (n = 55). The following information was obtained for all patients: socio-demographic data, number of blood transfusions and some laboratory investigations. After 39.45 ± 7.57 mo follow-up, HBV markers were measured in these patients by CMI.

RESULTS: The incidence of HBV infection in maintenance hemodialysis patients was 37.5%, which was higher than in controls (9%). In the patients positive for HBV markers, there were 13 patients (39.4%) who had a history of blood transfusion, which was more than the number [12 (21.8%), P = 0.04] of patients negative for HBV markers. Eight of the 88 patients negative for HBV markers turned out to be positive, while three of the 33 patients positive for HBV markers turned out to be negative. There was no cirrhosis of the liver or hepatoma occurring in these patients.

CONCLUSION: Maintenance hemodialysis patients have a higher risk of HBV infection than the average population. The number of blood transfusions is associated with an increased prevalence of HBV. While it is hard for hemodialysis patients to eliminate HBV, the prognosis of patients with positive HBV markers is good.

Key words: Hepatitis B virus; Infection; Hemodialysis patients; Maintenance; Prevalence

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INTRODUCTION

Hepatitis B virus (HBV) infection remains a serious issue in the dialysis population[1-5]. Introduction of HBV vaccination, isolation of HBV positive patients, use of dedicated dialysis machines and regular surveillance for HBV infection has dramatically reduced the spread of HBV in this setting[6-10]. However, the frequency of HBV infection in patients undergoing maintenance dialysis in the industrialized world is low, but not negligible[11-14]. Persistent HBsAg seropositivity is much higher in less-developed countries[15-19], especially in China. The prevalence of HBV infection among hemodialysis patients varies between countries and between dialysis units within a single country. The present study was undertaken to investigate the prevalence of HBV infection among maintenance hemodialysis patients.

MATERIALS AND METHODS

Patients

Eighty-eight patients with ESRD on long-term hemodialysis in the center of blood purification of Beijing Chaoyang Hospital were recruited for this study. There were 42 male and 46 female patients. The median age was 55.46 ± 13.78 (range 25-76) years. The primary cause of ESRD was established in these patients: chronic glomerulonephritis in 35 patients (39.8%), diabetic nephropathy in 22 (25%), hypertension nephropathy in 19 (21.5%), glomerulopathy of unknown origin in 4 (4.5%), tubulointerstitial nephritis in 2 (2.3%), polycystic kidney

Table 1 Variations of HBV markers between hemodialysis patients and healthy controls

Hemodialysis patients	Volunteer controls	HBsAg	Anti-HBs	Anti-HBc	HBeAg	Anti-HBe
18	0			+		
15	2		+	+		
7	0		+	+		+
1	0			+		+
2	1	+		+	+	
0	6	+				

disease in 2 (2.3%), and renal cell carcinoma in 2 (2.3%). All patients had been on dialysis for 39.45 ± 7.57 (range from 36 to 49) mo. Hemodialysis was performed two to three times each week, 4-4.5 h per session, using single-use dialyzers with a membrane surface area of 1.3-1.7 m^2. Dialysis membranes were made of polysulfone (36.7%), cellulose acetate (25.4%), or polymethyl-metacrylate (37.9%).

The control group was made up of 100 healthy blood donors and hospital staff [52 males and 48 females, with a median age of 47.25 ± 10.10 (range 35-69) years], whose health status was assessed by periodical general check-ups. These were healthy persons without any infectious, hepatic or kidney diseases.

Methods

Before each patient entered into our blood purification unit, HBV markers were measured, including hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis B e antigen (HBeAg), and antibody to hepatitis B e antigen (anti-HBe). These markers were also measured in healthy controls. There were 33 (37.5%) patients positive for HBV markers, i.e. patients positive for HBsAg, anti-HBc, HBeAg, anti-HBe, with or without positive anti-HBs. We therefore divided all patients into two groups: patients positive for HBV markers (n = 33) and patients negative for HBV markers (including those only positive for anti-HBs) (n = 55).

Serum HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe were measured with chemiluminescent microparticle immunoassay (CMI) using an ARCHITECT immunoassay analyzer from the Diagnostics Division of Abbott Laboratories (Abbott Park, IL, United States). Biochemistry data were determined using an AU500 autoanalyzer for blood urea nitrogen (BUN), creatinine, serum albumin, alanine aminotransferase (ALT), aspirate aminotransferase (AST) and alkaline phosphatase (ALP).

RESULTS

Incidence of HBV in maintenance hemodialysis patients

There were thirty-three patients with positive HBV markers at the beginning of the study. The incidence of HBV in maintenance hemodialysis patients was 37.5%, which was significantly higher than that in healthy controls. Nine controls were positive for HBV markers. The incidence of HBV in controls was 9%. The variation of the two groups is shown in Table 1.

Table 2 Clinical characteristics and relevant laboratory data (mean ± SD)

	Positive HBV markers group n = 33	Negative HBV markers group n = 55	P value
Time on HD, months	38.45 ± 9.34	40.55 ± 8.53	0.245
Blood transfusion	13 (39.4%)	12 (21.8%)	0.042
Biochemical data			
BUN, mg/dL	59.53 ± 12.36	56.47 ± 11.43	0.755
Creatinine, mg/dL	59.53 ± 12.36	56.47 ± 11.43	0.755
Serum albumin, g/dL	3.6 ± 0.6	3.7 ± 0.5	0.206
AST, IU/L	26.3 ± 6.9	23.6 ± 7.4	0.052
ALT, IU/L	18.7 ± 8.3	16.5 ± 10.2	0.235
ALP, IU/L	76 ± 15.7	79 ± 13.6	0.072

BUN: Blood urea nitrogen, detected Pre-dialysis; AST: Asparate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; HD: Hemodialysis.

Risk factors of HBV infection

The basic clinical characteristics of the patients are shown in Table 2. In the negative HBV marker group, the mean time on hemodialysis was longer than for the positive HBV marker group, but this difference was not statistically significant. The AST, ALT and ALP levels were, as expected, higher in the positive HBV marker group. The levels of BUN, creatinine, and serum albumin were similar in these two groups. In the positive HBV marker group, there were thirteen patients (39.4%) with a history of blood transfusion, more than the negative group [12 patients (21.8%), P = 0.04].

Turnover and prognosis of the two groups

There were twenty-eight patients positive for HBV markers at the beginning of the study. Three of these became negative, such that the rate was 5.4% (1.8% per year). There were 60 patients negative for HBV markers at baseline, eight of whom turned out to be positive, for a rate of 12.7% (4.2% per year). 39.45 ± 7.57 mo later, chronic cirrhosis and hepatoma had not occurred in these patients.

DISCUSSION

This work shows a high prevalence of HBV infection (37.5%) in maintenance hemodialysis patients (Table 1). The prevalence of HBV infection in hemodialysis patients was higher than that of normal controls (9%) and the general population (9.09%)[20]. This may be attributed to China's high endemic state for HBV infection. A potential contributor to this phenomenon is that significant cellular immune disturbances typically occur in hemodialysis patients. Sharing of supplies, instruments, or medications between hemodialysis patients and reuse of dialyzers would in theory increase the spread of HBV between patients. China, a country hyperendemic for HBV infection, has a higher rate of HBV infection than most industrialized nations. That is why the prevalence rate of HBV infection in our hemodialysis patients is higher than previous observations from western countries (0.9%-5.9%)[11-14] as well as from other developing nations (4%-17%)[15-19].

In order to study the risk of HBV infection, we examined the time on hemodialysis, the biochemical data and the history of blood transfusions of the two groups of patients. We conclude that there is an association between blood transfusions and the prevalence of HBV infection. However, there was no significant difference between time on hemodialysis and HBV infection. In our research, although AST, ALT and ALP levels were higher in the positive HBV marker group, this difference was not statistically significant. As for hemodialysis patients, Hung *et al*[21] revised cutoff values for AST (24 IU/L) and ALT (17 IU/L), which had better sensitivities. In the HBV infected group, the mean values of AST (26.3 IU/L) and ALT (18.7 IU/L) exceeded this criteria, which have important clinical implications in providing benefits of earlier detection and possible prevention of chronic hepatic deteriorations[22].

Because of cellular immune status disturbances, it is hard for hemodialysis patients to eliminate HBV[23-26]. In this study, three patients turned out to be negative, giving a rate of 5.4% (1.8% per year). As for the patients negative for HBV markers, eight turned out to be positive, with a rate of 12.7% (4.2% per year). HBV-related liver disease in patients on long-term dialysis often appears clinically mild, with only modest elevations in AST and ALT levels. Few studies have addressed the natural history of HBV in the dialysis population. Josselson *et al*[27] reported no significant differences in death rates, hospitalizations and hospitalized days between HBsAg-positive and -negative patients on maintenance hemodialysis in the US. However, different outcomes were noted in a retrospective study from India[28]. HBsAg positive patients had a significantly higher mortality rate than negative patients. In our study, the development of cirrhosis, hepatoma and decompensation of liver function is not observed in HBV infected hemodialysis patients. It has been suggested that the hemodialysis procedures lower HBV DNA levels by various mechanisms: the clearance of HBV DNA by the dialysate, the entrapment of HCV DNA particles onto the membrane surface of dialyzers, and the production of cytokines and other substances during hemodialysis sessions. Rampino *et al*[29] have measured a prolonged and marked production of hepatocyte growth factor (HGF) during hemodialysis sessions, and have suggested a beneficial effect of HGF through hepatocyte proliferation and accelerated liver repair. Badalamenti *et al*[30] observed that IFN-alpha levels markedly increase after dialysis using both cellulose and synthetic membranes. This increase in endogenous IFN could contribute to a reduction in viremia in HBsAg patients on maintenance dialysis.

In conclusion, the incidence of HBV in maintenance hemodialysis patients is significantly higher than the average population. The number of blood transfusions is associated with an increased prevalence of HBV. While it is hard for hemodialysis patients to eliminate HBV, the prognosis for patients with positive HBV markers is good.

COMMENTS

Background

Sharing of supplies, instruments, or medications between hemodialysis patients and reuse of dialyzers would in theory increase the spread of HBV between patients. Persistent HBsAg seropositivity is much higher in China than in other countries. In order to get the exact prevalence rate of hepatitis B virus (HBV) infection in maintenance hemodialysis patients, we investigate a dialysis unit in China.

Research frontiers

We studied eighty-eight hemodialysis patients who had been regularly receiving hemodialysis for an average of 39.45 ± 7.57 mo. We measured those patients' HBV markers before hemodialysis and after 39.45 ± 7.57 mo follow-up. We get the prevalence of HBV infection in maintenance hemodialysis patients.

Innovations and breakthroughs

Firstly, this work shows a high prevalence of HBV infection (37.5%) in maintenance hemodialysis patients. Secondly, it concludes that there is an association between blood transfusions and the prevalence of HBV infection. However, there was no significant difference between time on hemodialysis and HBV infection. Thirdly, the main difference from other related articles is that we find three positive HBV-infected patients turned out to be negative.

Applications

The actual application value: the prevalence of HBV infection in maintenance hemodialysis patients in China. The perspective of future application: the further study for the exact mechanisms as to how the hemodialysis patients eliminate HBV.

Terminology

Maintenance hemodialysis patients: The patients who suffer from end-stage renal disease have to receive regular hemodialysis.

Peer review

The authors have estimated the prevalence of HBV infection in a hemodialysis unit. It is concluded that maintenance hemodialysis patients have a higher risk of HBV infection than the average population. The number of blood transfusions is associated with an increased prevalence of HBV. While it is hard for hemodialysis patients to eliminate HBV, the prognosis of patients with positive HBV markers is good.

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RAPID COMMUNICATION

Decreased expression of serotonin in the jejunum and increased numbers of mast cells in the terminal ileum in patients with irritable bowel syndrome

Shen-Hao Wang, Lei Dong, Jin-Yan Luo, Jun Gong, Lu Li, Xiao-Lan Lu, Shui-Ping Han

Shen-Hao Wang, Lei Dong, Jin-Yan Luo, Jun Gong, Lu Li, Xiao-Lan Lu, Department of Gastroenterology, Second Hospital of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China
Shui-Ping Han, Department of Pathology, Medical School of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Supported by the Key Clinical Project (2004) from the National Ministry of Health, No. 2004-56
Correspondence to: Professor Lei Dong, Department of Gastroenterology, Second Hospital of Xi'an Jiaotong University, 157, Xi 5 Road, Xi'an 710004, Shaanxi Province, China. donglei4488@sina.com
Telephone: +86-29-87679272
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Abstract

AIM: To investigate if there are changes in serotonin (5-HT) levels, enterochromaffin (EC) cells and mast cells in small intestinal mucosa of patients with irritable bowel syndrome (IBS).

METHODS: Diarrhea-predominant (IBS-D, *n* = 20), or constipation-predominant (IBS-C, *n* = 18) IBS patients and healthy controls (*n* = 20) underwent colonoscopy and peroral small intestinal endoscopy, and mucosal samples were obtained at the descending part of the duodenum, proximal end of jejunum and terminal ileum. High-performance liquid chromatography-electrochemistry and immunohistochemical methods were used to detect 5-HT content, EC cells and mast cells.

RESULTS: (1) There were no differences in the number and distribution of EC cells between IBS patients and the normal group. (2) The mucosal 5-HT contents at the duodenum, jejunum and ileum in IBS-C patients were 182 ± 90, 122 ± 54, 61 ± 35 ng/mg protein, respectively, which were all lower than those in the normal group (256 ± 84, 188 ± 91, and 93 ± 45 ng/mg protein, respectively), with a significant difference at the jejunum ($P < 0.05$). There were no differences in the small intestinal mucosal 5-HT contents between IBS-D patients and the normal group. The mucosal 5-HT contents at the duodenum were significantly higher than those at the ileum in the three groups ($P < 0.001$). (3) The numbers of mast cells in patients with IBS-C and IBS-D at the ileum were 38.7 ± 9.4 and 35.8 ± 5.5/high power field (hpf), respectively, which were significantly more than that in the normal group (29.8 ± 4.4/hpf) ($P < 0.001$). There was no significant difference in the numbers of mast cells at the other two parts between IBS patients and the normal group. The numbers of mast cells in IBS-C, IBS-D, and normal groups were all significantly higher at the ileum (38.7 ± 9.4, 35.8 ± 5.5, 29.8 ± 4.4/hpf, respectively) than at the duodenum (19.6 ± 4.7, 18.5 ± 6.3, 19.2 ± 3.3/hpf, respectively, $P < 0.001$).

CONCLUSION: The changes in the 5-HT signaling pathway at the jejunum of IBS-C patients and the increase in mast cells in patients with IBS at the terminal ileum may offer evidence to explain the pathogenesis of IBS.

Key words: Enterochromaffin cell; Irritable bowel syndrome; Mast cell; Serotonin; Small intestinal mucosa

Wang SH, Dong L, Luo JY, Gong J, Li L, Lu XL, Han SP. Decreased expression of serotonin in the jejunum and increased numbers of mast cells in the terminal ileum in patients with irritable bowel syndrome. *World J Gastroenterol* 2007; 13(45): 6041-6047

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INTRODUCTION

Irritable bowel syndrome (IBS) has a very high global incidence nowadays[1,2]. Though it is not fatal, it exerts a strong influence on the quality of human life[3]. Preliminary epidemiological surveys in China had the same suggestions[4,5].

The pathogenesis of IBS is not clear. Studies have found that alterations of many elements in the gastrointestinal mucosa serotonin (5-HT) signaling pathway occurs in IBS patients[6], and this was one of the mechanisms for the disrupted visceral sensation and gastrointestinal motility in IBS patients. It has been shown that mast cells also play a role in the visceral hypersensitivity of IBS patients. Thus, 5-HT content in the mucosa, enterochromaffin cells (EC cells) and mast cells are the most important indicators. Thus far, many studies

on 5-HT content, EC cells and mast cells have been carried out based on samples taken from the ileocecum, colon and rectum. It is not clear whether the small intestinal mucosa 5-HT content, EC cells and mast cells contribute to the pathological and physiological mechanism in IBS patients. The purpose of this study was to compare 5-HT content, the distribution and number of EC cells and mast cells in small intestinal mucosa in order to determine if there are changes in the 5-HT signaling pathways and mast cells in IBS patients.

MATERIALS AND METHODS

Subjects

Following the Rome Ⅲ Criteria[7], 38 IBS patients were selected as subjects, all of whom were outpatients in Department of Gastroenterology, The Second Hospital of Xi'an Jiaotong University from July 2006 to February 2007. Among them, 20 patients (8 male, 12 female, aged 48.7 ± 16.6 years) had IBS-D and 18 IBS-C (9 male, 9 female, aged 41.5 ± 15.1 years). These patients all underwent testing and examinations of their blood, feces, liver function and fasting blood-glucose levels. A colonoscopy and other related procedures were also performed so as to exclude organic diseases. The patients were carefully interviewed about their situations before the onset of the disease to see if they had had any attacks of acute gastroenteritis infectiosa. Those who had were regarded as post-infection IBS (PI-IBS). In this study, the number of PI-IBS samples gathered were not sufficient so they were not included in the analysis and discussion. 20 healthy people were selected as the normal group (9 male, 11 female, aged 39.9 ± 19.5 years). 5 of them underwent examinations because they were suspected of developing malignant diseases caused by familial heredity. 10 of them came to the hospital for reexamination because of intestinal polypi. 5 were volunteers. All the people in the normal group were found to be clear of a history of chronic diseases or symptoms of gastrointestinal diseases such as abdominal pain, diarrhea and constipation. Written consent was obtained from each subject. The study was approved by the Ethics Committee of the Second Hospital of Xi'an Jiaotong University and conducted according to the principle of the Declaration of Helsinki in 1995.

Samples

All the subjects stopped administering any drugs or treatments which might affect the gut movement for at least a week prior to the examinations. The first examination they had was colonoscopy (Pentex EC 3830F) and 6 pieces of mucosa from the terminal ileum, 15 cm from the ileocecal valve, were taken at the same time. After 1 or 2 d for rest, they underwent peroral small intestinal endoscopy (Fujinon EN 450P) and 12 pieces of mucosa were taken, 6 from the descending part of the duodenum, 6 from the proximal end of the jejunum, 15 cm from the ligament of Traitz. For each kind of piece of mucosa, 2 pieces were immediately put into 40 g/L formaldehyde fixatives for use later in immunohistochemistry. Four pieces were put into the plastic tubes and preserved in a refrigerator at -80℃.

Immunohistochemical staining

The mucosa samples were paraffin-embedded in the typical manner and stained with immunohistochemical-SP. For EC cells and mast cells, rabbit anti-human 5-HT antibody (Zhongshan Jinqiao Company, Beijing, Product No. ZA-0231, dilution 1:100) and mouse anti-human tryptase antibody (Maixin_Bio Company, Fuzhou, Product No. MAB-0125, dilution 1:100) were used as primary antibodies. The secondary antibody staining kit was SP9000 of broad spectrum provided by the Zhongshan Jinqiao Company, Beijing. Next, the samples were observed under a powerful optical microscope (× 400). Each piece of mucosa was observed continuously from 6 non-overlapping fields of view and the numbers of positive immunoreactive cells were counted, with each number expressed with "mean ± SD".

Measurement of mucosal 5-HT content

300 μL 0.2 mol/L perchloric acid containing EDTA was added to the small intestinal mucosa samples prior to homogenation. 50 μL of centrifuged supernatant fluid was sent to the Xi'an Institute for Drug Control for determination of the 5-HT content with high-performance liquid chromatography-electrochemistry (HPLC-ECD), which was expressed in ng/mg protein.

Statistical analysis

One-way analysis of variance (ANOVA) was conducted with the SPSS 13.0 software to compare 5-HT content and the number of EC cells and mast cells between the three groups. $P < 0.05$ indicated significant differences.

RESULTS

EC cells

Under optical microscopy, EC cells were distributed in the intestinal gland cavity, mainly in intestinal crypts. They were next to the goblet cells and most of them were in the shape of a cone or rhombus-like. For each field of view under a powerful optical microscope (× 400), the number of EC cells at the descending part of the duodenum in patients with IBS-C and IBS-D was 9.4 ± 3.9/high power field (hpf) and 10.2 ± 3.7/hpf, respectively, 6.7 ± 2.6/hpf and 6.2 ± 2.4/hpf at the proximal end of the jejunum, 2.7 ± 1.4/hpf, 3.2 ± 1.9/hpf at the terminal ileum. Compared with those in the normal group (10.5 ± 3.4/hpf, 6.6 ± 3.4/hpf and 3.1 ± 1.7/hpf, respectively), there were no significant differences ($P > 0.05$, Figure 1). The distribution of EC cells in the small intestinal mucosa in patients with IBS-C and IBS-D was similar to that in the normal group, proportionately decreasing from the descending part of the duodenum to the terminal ileum. In all three groups, the number of EC cells at the descending part of the duodenum was significantly different from that at the terminal ileum ($P < 0.001$). The number of EC cells at the proximal end of the jejunum was significantly less than that at the descending part of the duodenum ($P < 0.05$) and significantly more than that at the terminal ileum ($P < 0.01$). Figure 2 shows the EC cells after staining under a powerful microscope.

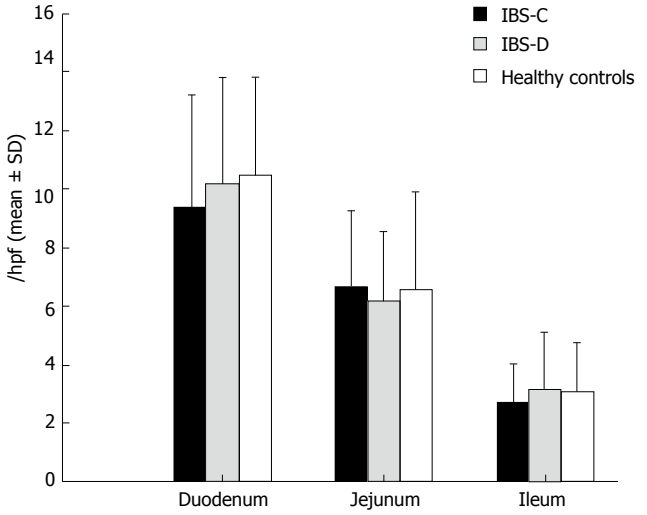

Figure 1 The number of small intestinal mucosal EC cells in IBS patients and the healthy controls. The number and distribution of EC cells in IBS patients are similar to those in the healthy controls. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS; hpf; High power field.

5-HT content

The 5-HT content in IBS-C patients at the descending part of the duodenum, proximal end of the jejunum and terminal ileum were 182 ± 90 ng/mg protein, 122 ± 54 ng/mg protein and 61 ± 35 ng/mg protein, respectively, which were all lower than that in the normal group (256 ± 84 ng/mg protein, 188 ± 91 ng/mg protein and 93 ± 45 ng/mg protein, respectively). In addition, the 5-HT content at the proximal end of the jejunum had a significant difference compared with that of the normal group ($P < 0.05$). The P value at the descending part of the duodenum was 0.058, and at the terminal ileum, 0.063. The 5-HT contents in IBS-D patients at the descending part of the duodenum, proximal end of the jejunum and terminal ileum were 220 ± 96 ng/mg protein, 167 ± 58 ng/mg protein and 70 ± 41 ng/mg protein, respectively, which were all lower than those in the normal group, but they did not show statistical significance ($P > 0.05$). Of all the three groups, the 5-HT content at the descending part of the duodenum was significantly higher than that at the terminal ileum ($P < 0.001$), with the 5-HT content at the jejunum falling between them (Figure 3).

Mast cells

Under optical microscopy, mast cells were distributed in the lamina propria, in the shape of an egg or ellipse. They were scattered between the mucous glands with brown cytoplasm. For each field of view under a powerful optical microscope (× 400), the number of mast cells in patients with IBS-C and IBS-D at the terminal ileum were 38.7 ± 9.4/hpf and 35.8 ± 5.5/hpf, respectively, which were significantly more than those in the normal group (29.8 ± 4.4/hpf) ($P < 0.001$). However, the numbers of mast cells in patients with IBS-C and IBS-D did not show significant differences ($P > 0.05$). The number of mast cells at the descending part of the duodenum in patients with IBS-C and IBS-D were 19.6 ± 4.7/hpf and 18.5 ± 6.3/hpf, respectively, and 18.8 ± 5.8/hpf and 19.7 ± 4.8/hpf at the proximal end of the jejunum. Compared with those in the normal group (19.2 ± 3.3/hpf and 20.0 ± 6.9/hpf, respectively), they did not have significant differences ($P > 0.05$, Figure 4). The number of mast cells in the three groups at the ileum were all significantly more than those at the duodenum ($P < 0.001$). However, the numbers of mast cells at the jejunum and at the duodenum did not show significant differences ($P > 0.05$). Figure 5 shows the mast cells after staining under a powerful microscope.

DISCUSSION

The pathological mechanism of IBS is not clear[8]. It is thought that it is associated with alterations in mentality, GI motility, visceral sensitivity, *etc*. The abnormality in the 5-HT content is one of the reasons for the visceral hypersensitivity and gastrointestinal dysmotility of IBS patients[9], and the mast cells have something to do with the visceral hypersensitivity of IBS patients[10]. Because of the difficulty and trouble in taking samples from intestines, many studies on IBS so far have been based on research in mucosa from the ileocecum, colon and rectum, rather than the whole small intestine. To our knowledge, this study is a pioneering one that aims to determine the 5-HT content and number of EC cells and mast cells in the small intestinal mucosa, esp. duodenum and jejunum, in IBS patients. It is also a first study selecting subjects by following Rome Ⅲ Criteria. In order to better understand the mechanism of IBS, patients were divided into two groups, i.e. patients without any attacks of acute gastroenteritis infectiosa before the onset of the disease, and those with PI-IBS, although the number was not sufficient for the analysis.

5-HT is a very important neurotransmitter of the digestive tract; it is essential to the brain-gut connection and related to gastrointestinal motility and visceral sensation. Most of the 5-HT in the digestive tract is stored in EC cells. When EC cells are stimulated, they release 5-HT, which will act upon 5-HT receptors in intestinal nerve fibers and smooth muscle, initiate peristaltic, secretory, vasodilatory, vagal, and nociceptive reflexes, or regulate sensory function by way of vagal spinal afferent nerves. The serotonin-selective reuptake transporter (SERT) terminates the physiological function of 5-HT by taking it back up in the mucosa[11]. When studying the 5-HT signaling pathway, important elements to examine include the number of EC cells, 5-HT content, tryptophan hydroxylase level, 5-hydroxyindoleacedic acid (HIAA), plasma 5-HT concentration and SERT expression[12]. Studies on animals and humans have measured the above elements in gastric and small intestinal mucosa and drawn some meaningful conclusions with regard to the mucosa 5-HT signaling pathway[13-15]. In this study, two important indicators, EC cells and 5-HT content, were used to study the small intestinal 5-HT signaling pathway in IBS patients.

The clinical symptoms of IBS patients include abdominal pain, diarrhea and constipation. 5-HT is closely related to gastrointestinal motility and visceral sensation. Therefore, abnormalities in the 5-HT signaling pathway are regarded as the cause of visceral hypersensitivity, gastrointestinal dysmotility and parasecretion in IBS

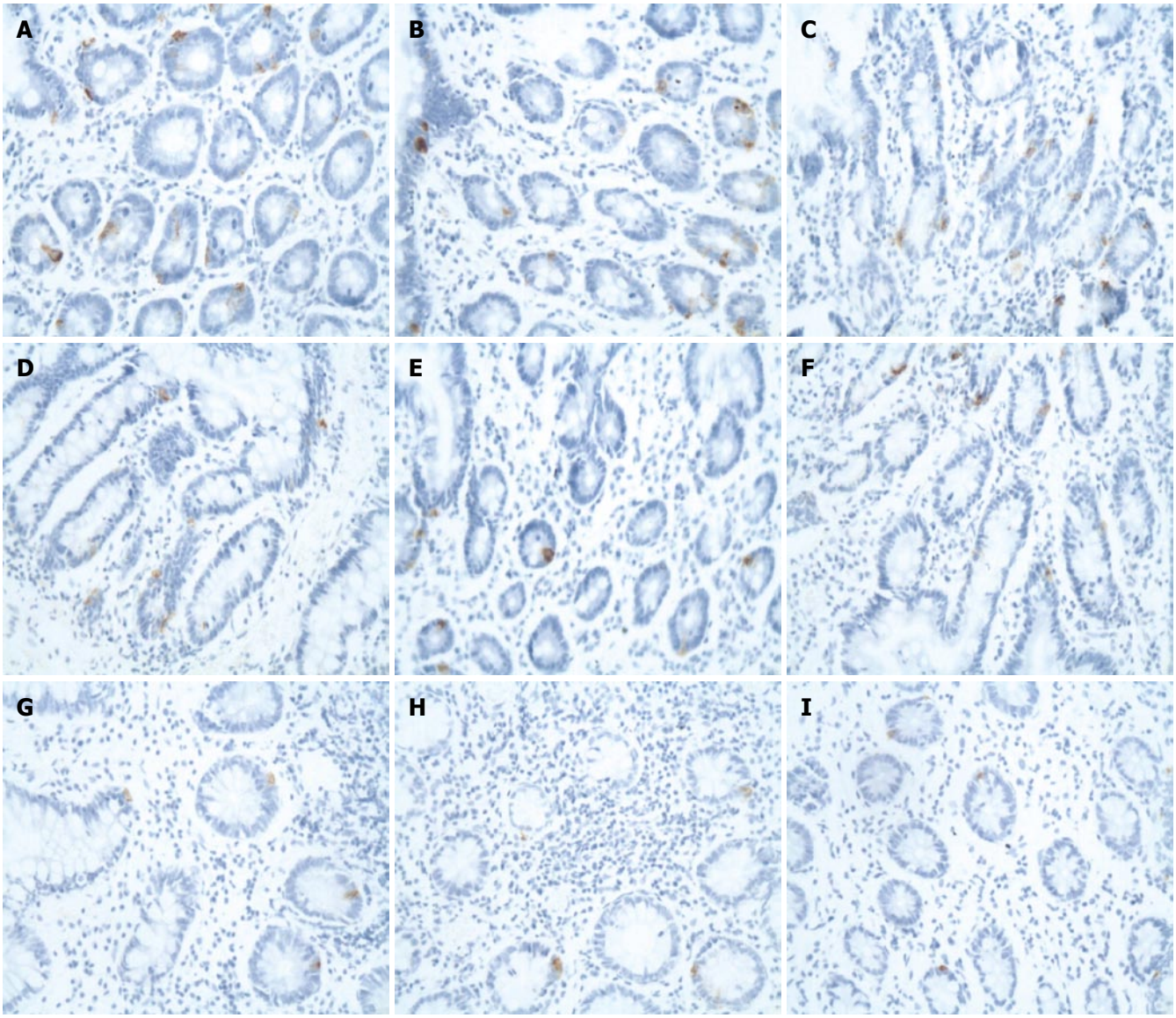

Figure 2 EC cells after being stained by rabbit anti-human 5-HT antibody under a powerful microscope (× 400). **A-C**: EC cells at the descending part of duodenum in healthy controls, IBS-C patients and IBS-D patients, respectively; **D-F**: EC cells at the proximal end of the jejunum in healthy controls, IBS-C patients and IBS-D patients, respectively; **G-I**: EC cells at the terminal ileum in healthy controls, IBS-C patients and IBS-D patients, respectively. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS.

patients. A study performed by Coates *et al*[12] suggested that the 5-HT signaling pathway in the mucosa of the rectum in patients with IBS-C and IBS-D had a molecular defect. However, thus far, there have been no studies on 5-HT in the whole small intestine mucosa in IBS patients.

Previous studies on 5-HT content in the colonic mucosa in IBS patients resulted in different conclusions. Coates *et al*[12] thought that the 5-HT content in the rectal mucosa in patients with IBS-C and IBS-D was significantly lower than that of the normal group. A study by Wang *et al*[16] in China suggested that although the 5-HT content in the colonic mucosa in IBS patients was lower than that of the normal group, it did not have statistical significance. In this study, the 5-HT content in various parts of the small intestinal mucosa in IBS-C patients were all decreased, and the 5-HT content at the proximal end of the jejunum was statistically different from that in the normal group ($P < 0.05$). Based on other previous studies, we propose that the decrease in 5-HT results in the weakening of various reflexes, decreasing of secretion and constipation. The fact that there was not much change in 5-HT content in the small intestinal mucosa in IBS-D patients suggested the existence of an abnormality in other 5-HT signaling pathways. Other studies draw different conclusions. Miwa *et al*[17] thought that the increased 5-HT content in colonic mucosa in IBS-C patients relative to normal controls and IBS-D patients suggested that the synthesis of 5-HT was normal, but the release of 5-HT was changed after EC cells were stimulated.

Other studies on EC cells in IBS patients came to different conclusions. The change in EC cells in the intestinal tract in IBS patients was first found in PI-IBS patients. The increase in EC cells was regarded as a characteristic change in PI-IBS patients[18-20], but it was not applied to IBS patients who had had no infection before the onset of the disease. Studies made by Dunlop

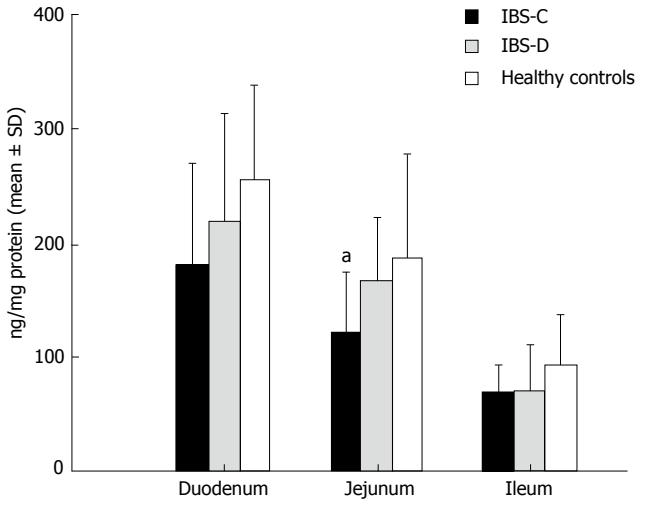

Figure 3 The content of small intestinal mucosal 5-HT in IBS patients and the healthy controls. For each part, the 5-HT content in the IBS-C patients is lower than that in the healthy controls. The 5-HT content at the proximal end of jejunum shows a significant difference compared with that in the normal group ($P < 0.05$). The 5-HT content in the three groups at the descending part of duodenum are all significantly higher than that at the terminal ileum ($P < 0.001$). $^aP < 0.05$ *vs* healthy controls. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS.

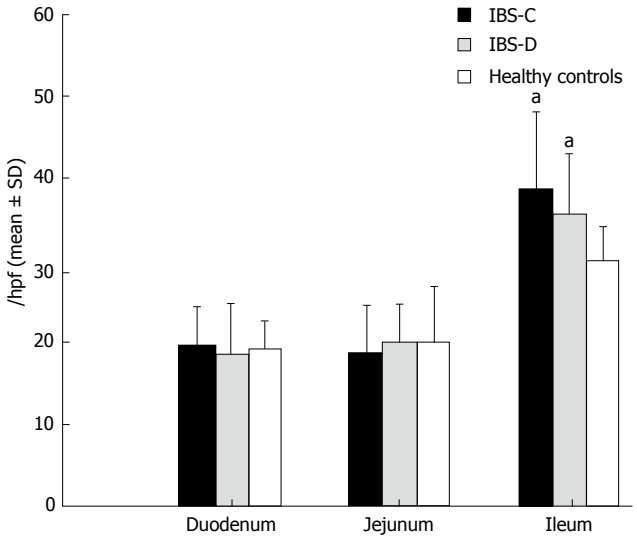

Figure 4 The number of small intestinal mucosal mast cells in IBS patients and the healthy controls. The number of mast cells at the terminal ileum in patients with IBS is significantly different compared with that in the healthy controls ($P < 0.001$). The numbers of mast cells in patients with IBS-C and IBS-D at the other two parts of the intestine do not have significant differences, compared with those in the healthy controls ($P > 0.05$). $^aP < 0.05$ *vs* healthy controls. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS; hpf: High power field.

et al[18,19] and Spiller *et al*[20] found no increase in EC cells in IBS patients with no infection before the onset of the disease. Coates *et al*[12] thought that there was no change in the number of EC cells in the rectal mucosa in patients with IBS-C and IBS-D. Li *et al*[21] and Jiang *et al*[22] found that the number of EC cells at the junction of the rectum and sigmoid colon in patients with IBS-C and IBS-D significantly increased, but in the ileocecum it did not. In this study, the number and distribution of EC cells in the small intestinal mucosa in IBS patients did not show significant differences compared with those in the normal group, which suggested there were no obvious pathological changes in the EC cells in the small intestinal mucosa in IBS patients.

Looking at the small intestinal mucosa 5-HT content and the distribution and number of EC cells in IBS patients, we found that the 5-HT content decreased but the number of EC cells remained unchanged compared with that in the normal group, which suggested that the amount of 5-HT released by EC cells in the small intestinal mucosa in IBS-C patients was less than that in the normal group. In IBS-D patients, the 5-HT content and number of EC cells remained the same as those in the normal group, suggesting a difference in 5-HT signaling pathways in IBS-C and IBS-D patients.

Studies have shown that mast cells have something to do with the visceral hypersensitivity of IBS patients[23]. Many agents released by mucosa mast cells can affect intestinal nerve and smooth muscle. Experiments on animals and studies in human beings all proved that mast cells and intestinal nerves are closely connected[24], and in IBS patients, the connection was even closer[25]. The increase of mast cells in IBS patients results in more agents being released by mast cells. All these, together with the close connection between the mast cells and nerve fibers, contribute to the seriousness of abdominal pain.

Many studies have demonstrated that the increase of mast cells in the ileocecum is a characteristic change of IBS[23,26,27]. Park *et al*[23], Dong *et al*[26] and Chen *et al*[27] found an increased number of mast cells in the ileocecum mucosa in IBS patients. Meanwhile, studies suggested the ileocecum might be the site of origin for abdominal pain, bloating, and changes in bowel habits, showing more sensitivity when a balloon dilates[28]. In this study, the number of mast cells at the terminal ileum in IBS patients increased significantly compared with that of the normal group, which is in line with the conclusions drawn from previous studies. It is indicated that the change of mast cell number in the terminal ileum in IBS patients is the characteristic pathological change. Studies on mast cells in other parts of the colonic mucosa (except the cecum) in IBS patients have produced different conclusions. Some found an increase in the number of mast cells[18,23,26], while others did not[19,26]. So far, in China, there have been no studies on mast cells in the duodenum and jejunum in IBS patients. In this study, the numbers of mast cells in the duodenum and jejunum in patients with IBS-C and IBS-D were almost the same as that in the normal group. This study also revealed that the distribution of mast cells in small intestinal mucosa in IBS patients was the same as that in the normal group, i.e. gradually increasing from the duodenum to the ileum.

In conclusion, this study reveals, for the first time, a change in the 5-HT signaling pathway in the jejunum in patients with IBS-C. It also suggests that the increase of mast cells in the ileocecum is the characteristic pathological change in IBS patients. These changes in the mucosa of gastrointestinal tract cause IBS-related symptoms. This

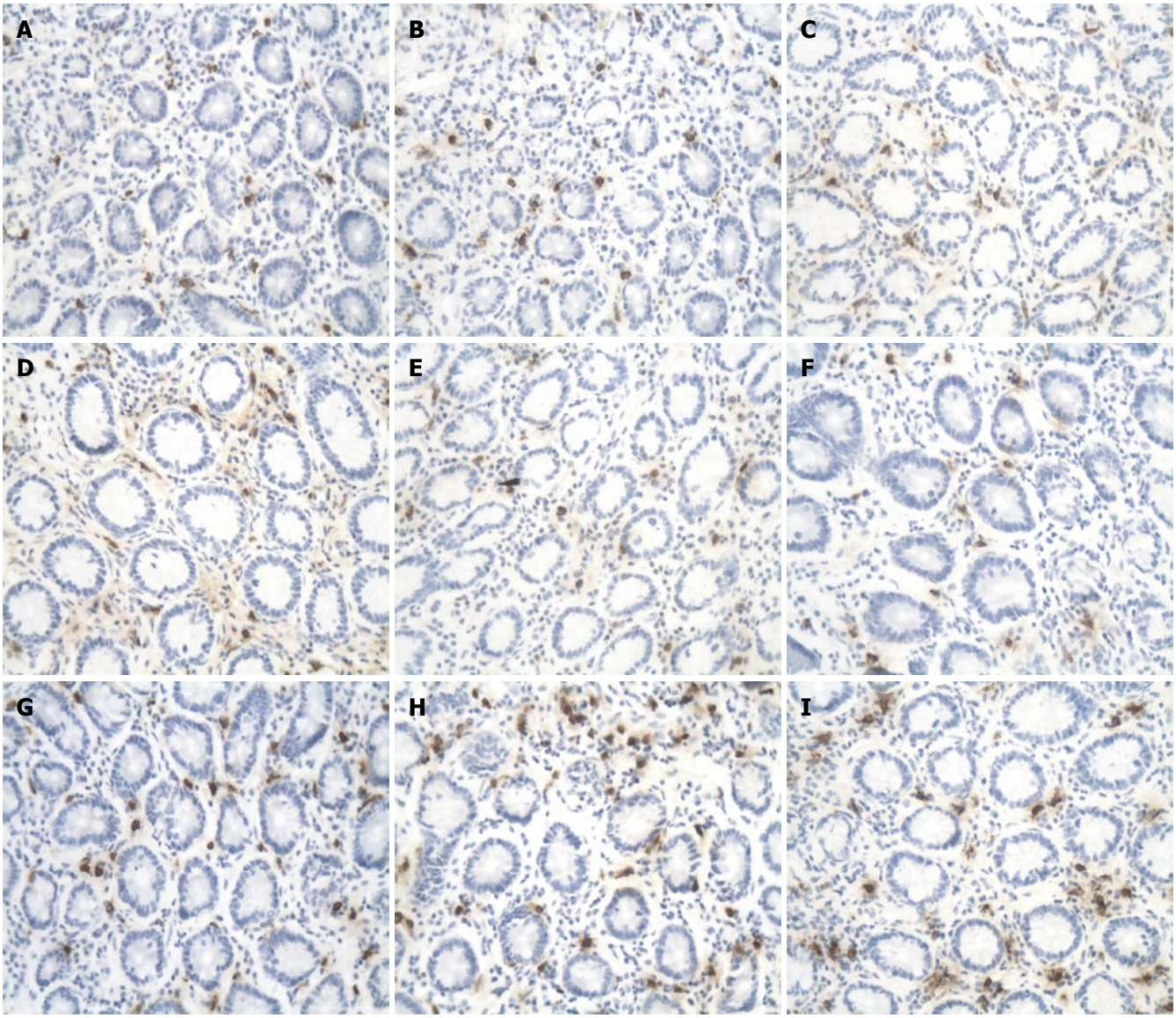

Figure 5 Mast cells after staining by mouse anti-human tryptase antibody under a powerful microscope (× 400). **A-C**: Mast cells at the descending part of the duodenum in healthy controls, IBS-C patients and IBS-D patients, respectively; **D-F**: Mast cells at the proximal end of jejunum in healthy controls, IBS-C patients and IBS-D patients, respectively; **G-I**: Mast cells at the terminal ileum in healthy controls, IBS-C patients and IBS-D patients, respectively. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS.

study offers new insight that may be useful for further research into the pathogenesis of IBS.

COMMENTS

Background

Studies have found that abnormal serotonin (5-HT) levels and mast cells are two of the reasons for the disturbance of visceral sensation and gastrointestinal motility in patients with irritable bowel syndrome (IBS). Many studies on 5-HT contents, enterochromaffin (EC) cells and mast cells in IBS have been carried out based on samples taken from mucosa from the ileocecum, colon and rectum, rather than in the whole small intestine.

Research frontiers

The purpose of this study was to compare 5-HT content, the distribution and number of EC cells and mast cells in small intestinal mucosa in order to determine if there were changes in 5-HT signaling pathways and mast cells in small intestinal mucosa in IBS patients.

Innovations and breakthroughs

(1) This study is the first one, to our knowledge, to determine the 5-HT contents, EC cells and mast cells in the small intestinal mucosa, especially the duodenum and jejunum, in IBS patients. (2) It is also the first study on the selection of subjects following Rome Ⅲ Criteria. (3) In order to better understand the mechanism of IBS, patients were divided into two groups; i.e. patients without any attack of acute gastroenteritis infectiosa before the onset of IBS and patients with previous gastrointestinal (GI) infections (post-infection IBS or PI-IBS) although, they could not be used as a group for the analysis due to the small number of cases.

Applications

These changes of 5-HT signaling pathway and mast cells in mucosa of the GI tract will cause IBS-related symptoms. This study offers new insight towards further research into the pathogenesis of IBS.

Terminology

Visceral hypersensitivity: When the GI tract is stimulated by luminal distention and other stimuli, perception of abdominal pain or discomfort is increased. This is widely regarded as the reason for the development of functional gastrointestinal diseases, including functional dyspepsia and irritable bowel syndrome. 5-HT signaling pathways: This refers to the whole process including 5-HT release in the GI tract and central nervous system, binding to its receptors, re-uptake and degradation. Elements involved in the study of the 5-HT signaling pathway include determination of the number of EC cells, 5-HT content, tryptophan hydroxylase

level, 5-hydroxyindoleacedic acid (HIAA), plasma 5-HT concentration and serotonin-selective reuptake transporter (SERT) expression.

Peer review

The study is of interest and permits a better understanding of IBS. It shows that the changes of 5-HT signaling pathway at the jejunum of IBS-C patients and the increase of mast cells in patients with IBS at ileocecum may offer evidence to explain the pathogenesis of IBS.

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RAPID COMMUNICATION

Prognostic value of lateral lymph node metastasis for advanced low rectal cancer

Ze-Yu Wu, Jin Wan, Jing-Hua Li, Gang Zhao, Yuan Yao, Jia-Lin Du, Quan-Fang Liu, Lin Peng, Zhi-Du Wang, Zhi-Ming Huang, Hua-Huan Lin

Ze-Yu Wu, Jin Wan, Gang Zhao, Yuan Yao, Jia-Lin Du, Quan-Fang Liu, Lin Peng, Zhi-Du Wang, Zhi-Ming Huang, Department of General Surgery, Guangdong Provincial People's Hospital, Guangzhou 510080, Guangdong Province, China
Jing-Hua Li, Department of Clinical Laboratory, The First Affiliated Hospital Huangpu Division, Sun Yat-Sen University, Guangzhou 510089, Guangdong Province, China
Hua-Huan Lin, Department of Pathology, Guangdong Provincial People's Hospital, Guangzhou 510080, Guangdong Province, China
Supported by the WST Foundation of Guangdong Province, No. 2000112736580706003
Correspondence to: Dr. Jin Wan, Department of General Surgery, Guangdong Provincial People's Hospital, Guangzhou 510080, Guangdong Province, China. reiqi123@sohu.com
Telephone: +86-20-83827812-60821 Fax: +86-20-83827812
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Abstract

AIM: To evaluate the risk factors for lateral lymph node metastasis in patients with advanced low rectal cancer, in order to make the effective selection of patients who could benefit from lateral lymph node dissection, as well as the relationship of lateral lymph node metastasis with local recurrence and survival of patients with advanced low rectal cancer.

METHODS: A total of 96 consecutive patients who underwent curative surgery with lateral pelvic lymphadenectomy for advanced lower rectal cancer were retrospectively analyzed. The relation of lateral lymph node metastasis with clinicopathologic characteristics, local recurrence and survival of patients was identified.

RESULTS: Lateral lymph node metastasis was observed in 14.6% (14/96) of patients with advanced low rectal cancer. Lateral lymph node metastasis was detected in 10 (25.0%) of 40 patients with tumor diameter $\geqslant$ 5 cm and in 4 (7.1%) of 56 patients with tumor diameter < 5 cm. The difference between the two groups was statistically significant (χ^2 = 5.973, P = 0.015). Lateral lymph node metastasis was more frequent in patients with 4/4 diameter of tumor infiltration (7 of 10 cases, 70.0%), compared with patients with 3/4, 2/4 and 1/4 diameter of tumor infiltration (3 of 25 cases, 12.0%; 3 of 45 cases, 6.7%; 1 of 16 cases, 6.3%) (χ^2 = 27.944, P = 0.0001). The lateral lymph node metastasis rate was 30.0% (9 of 30 cases), 9.1% (4 of 44 cases) and 4.5% (1 of 22 cases) for poorly, moderately and well-differentiated carcinoma, respectively. The difference between the three groups was statistically significant (χ^2 = 8.569, P = 0.014). Local recurrence was 18.8% (18 of 96 cases), 64.3% (9 of 14 cases), and 11.0% (9 of 82 cases) in patients with advanced low rectal cancer, in those with and without lateral lymph node metastasis, respectively. The difference between the two groups was statistically significant (χ^2 = 22.308, P = 0.0001). Kaplan-Meier survival analysis showed significant improvements in median survival (80.9 ± 2.1 m, 95% CI: 76.7-85.1 m *vs* 38 ± 6.7 m, 95% CI: 24.8-51.2 m) of patients without lateral lymph node metastasis compared with those with lateral lymph node metastasis (log-rank, P = 0.0001).

CONCLUSION: Tumor diameter, infiltration and differentiation are significant risk factors for lateral lymph node metastasis. Lateral pelvic lymphadenectomy should be performed following surgery for patients with tumor diameter $\geqslant$ 5 cm. Lateral lymph node metastasis is an important predictor for local recurrence and survival in patients with advanced low rectal cancer.

Key words: Low rectal cancer; Lateral lymph node metastasis; Local recurrence; Prognosis

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INTRODUCTION

It is well known that rectal carcinoma is one of the most common carcinomas in China. Since total mesorectal excision was adopted as the standard treatment of patients with rectal carcinoma, improvements have been made in decreasing its local recurrence and prolonging survival of patients[1-8]. However, even having undergone radical resection with total mesorectal excision, about 5%-40% of patients with rectal carcinoma have local recurrence[9-14]. The survival of patients with advanced low rectal cancer

still remains poor. It was reported that lateral lymph node metastasis may be the most important factor for local recurrence and poor prognosis of advanced low rectal cancer[15-18]. In the current study, the data on 96 consecutive patients who underwent curative surgery with total mesorectal excision and lateral lymph node dissection for advanced low rectal cancer at the Department of General Surgery of Guangdong Provincial People's Hospital were retrospectively analyzed. The relationship of lateral lymph node metastasis with local recurrence was identified. The prognostic value of lateral lymph node metastasis for advanced low rectal cancer was also evaluated. Moreover, the risk factors for lateral lymph node metastasis and indications of lateral lymph node dissection remain unclear. Therefore, this study was to explore the risk factors for lateral lymph node metastasis in order to make effective selection of patients who could benefit from lateral pelvic lymphadenectomy. The relation of lateral lymph node metastasis with clinicopathologic characteristics of advanced low rectal cancer was analyzed.

MATERIALS AND METHODS

Patients and methods

A total of 96 consecutive patients who underwent curative surgery with total mesorectal excision and lateral lymph node dissection for advanced low rectal cancer at the Department of General Surgery of Guangdong Provincial People's Hospital were retrospectively analyzed. There were 46 men and 50 women, ranging in age from 25 to 86 years, with a mean age of 65 years. None of these patients received preoperative chemotherapy or radiotherapy. Twenty-one patients (21.9%) had a family history, 40 patients (41.6%) had a high cancer embryonic antigen (CEA) level and a tumor diameter $\geqslant$ 5 cm, 56 had a tumor diameter < 5 cm. According to the Ming's criteria, 42 tumors were classified as expansive type carcinoma, 54 tumors as infiltrative type carcinoma. Fifty-six patients (58.3%) had positive lymph node metastases and 36 patients (37.5%) had positive vessel cancerous emboli. Thirty patients had a poorly differentiated carcinoma, 44 patients had a moderately differentiated carcinoma, 22 patients had a well-differentiated carcinoma. Low anterior resection was performed in 68 patients and abdominal perineal resection in 28 patients. A total of 1776 lymph nodes were dissected from these 96 patients (average 18.5 lymph nodes per patient). Two pathologists who were blinded to the clinicopathological data observed the specimens independently.

Statistical analysis

Statistical analysis was performed by chi-square test to examine the association of lateral lymph node metastasis with clinicopathologic characteristics and local recurrence of advanced low rectal cancer. The relationship between lateral lymph node metastasis and survival in patients with advanced low rectal cancer was evaluated by Kaplan-Meier survival analysis and log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Correlation between lateral lymph node metastasis and clinicopathologic characteristics of advanced low rectal cancer

Lateral lymph node metastasis was observed in 14.6 (14/96) of patients with advanced low rectal cancer. Lateral lymph node metastasis was detected in 10 (25.0%) of 40 patients with tumor diameter $\geqslant$ 5 cm and in 4 (7.1%) of 56 patients with tumor diameter < 5 cm. The difference between the two groups was statistically significant (χ^2 = 5.973, P = 0.015). Lateral lymph node metastasis was more frequent in patients with 4/4 diameter of tumor infiltration (7 of 10 cases, 70.0%), compared with patients with 3/4, 2/4 and 1/4 diameter of tumor infiltration (3 of 25 cases, 12.0%; 3 of 45 cases, 6.7%; 1 of 16 cases, 6.3%) (χ^2 = 27.944, P = 0.000). The lateral lymph node metastasis rate for poorly, moderately and well differentiated carcinoma was 30.0% (9 of 30 cases), 9.1% (4 of 44 cases) and 4.5% (1 of 22 cases), respectively. The difference between the three groups was statistically significant (χ^2 = 8.569, P = 0.014). No significant correlation was found between lateral lymph node metastasis and other variables such as gender (χ^2 = 0.168, P = 0.682), age (χ^2 = 0.103, P = 0.749), family history (χ^2 = 0.430, P = 0.512), high CEA level (χ^2 = 0.468, P = 0.494), Ming's classification (χ^2 = 0.430, P = 0.512), lymph node metastases (χ^2 = 0.239, P = 0.625) and vessel cancerous emboli (χ^2 = 0.201, P = 0.654) (Table 1).

Correlation between lateral lymph node metastasis and local recurrence of advanced low rectal cancer

Local recurrence was 18.8% (18 of 96 cases), 64.3% (9 of 14 cases), and 11.0% (9 of 82 cases) in patients with advanced low rectal cancer and those with and without lateral lymph node metastasis The difference between the two groups was statistically significant (χ^2 = 22.308, P = 0.0001).

Correlation between lateral lymph node metastasis and survival in patients with advanced low rectal cancer

In a median follow-up period of 73 (range 16-90) mo, Kaplan-Meier survival analysis showed a significantly improved median survival (80.9 $\pm$ 2.1 m, 95% CI: 76.7-85.1 m *vs* 38 $\pm$ 6.7 m, 95% CI: 24.8-51.2 m) in patients without lateral lymph node metastasis compared with those with lateral lymph node metastasis. The difference between the two groups was statistically significant (log-rank, P = 0.0001) (Figure 1).

DISCUSSION

Lateral pelvic lymphadenectomy for advanced low rectal cancer is controversial[19-25]. In Japan, lateral pelvic lymphadenectomy is routinely performed for patients with advanced low rectal cancer, whereas it is not frequently performed in the Western countries[26-28]. In the current study, a retrospective analysis was performed in 96 patients with advanced low rectal cancer who underwent curative surgery with lateral lymph node dissection. The relations of lateral lymph node metastasis with clinicopathologic

Table 1 Relations between lateral lymph node metastasis and clinicopathologic characteristics of patients with advanced low rectal cancer

Variable	*n*	Lateral lymph node metastasis		
		Positive (%)	Negative (%)	χ^2/*P* value
Gender				
Male	46	6 (13.0)	40 (87.0)	
Female	50	8 (16.0)	42 (84.0)	$\chi^2 = 0.168/P = 0.682$
Age (yr)				
< 60	38	5 (13.2)	33 (86.8)	
⩾ 60	58	9 (15.5)	49 (84.5)	$\chi^2 = 0.103/P = 0.749$
Family history				
Yes	21	4 (19.0)	17 (81.0)	
No	75	10 (13.3)	65 (86.7)	$\chi^2 = 0.430/P = 0.512$
CEA level				
High	40	7 (17.5)	33 (82.5)	
Normal	56	7 (14.6)	49 (85.4)	$\chi^2 = 0.468/P = 0.494$
Superficial diameter (cm)				
< 5	56	4 (7.1)	52 (92.9)	
⩾ 5	40	10 (25.0)	30 (75.0)	$\chi^2 = 5.973/P = 0.015$
Diameter of infiltration				
1/4	16	1 (6.3)	15 (93.7)	
1/2	45	3 (6.7)	42 (93.3)	
3/4	25	3 (12.0)	22 (88.0)	
4/4	10	7 (70.0)	3 (30.0)	$\chi^2 = 27.944/P = 0.000$
Ming's classification				
Expansive	42	5 (11.9)	37 (88.1)	
Infiltrative	54	9 (16.7)	45 (83.3)	$\chi^2 = 0.430/P = 0.512$
Histologic differentiation				
Well	22	1 (4.5)	21 (95.5)	
Moderate	44	4 (9.1)	40 (90.9)	
Poorly	30	9 (30.0)	21 (70.0)	$\chi^2 = 8.569/P = 0.014$
Lymph node metastasis				
Positive	56	9 (16.1)	47 (83.9)	
Negative	40	5 (12.5)	35 (87.5)	$\chi^2 = 0.239/P = 0.625$
Vessel cancerous emboli				
Positive	36	6 (16.7)	30 (83.3)	
Negative	60	8 (13.3)	52 (86.7)	$\chi^2 = 0.201/P = 0.654$

CEA: Carcinoma embryonic antigen.

characteristics, local recurrence and survival of advanced low rectal cancer were analyzed.

In our study, lateral lymph node metastasis was observed in 14.6% (14/96) of patients with advanced low rectal cancer, showing a significant correlation with tumor diameter, infiltration and differentiation. Lateral lymph node metastasis was found in 10 (25.0%) of 40 patients with tumor diameter ⩾ 5 cm and in 4 (7.1%) of 56 patients with tumor diameter < 5 cm ($\chi^2 = 5.973$, $P = 0.015$). Lateral lymph node metastasis was more frequent in patients with 4/4 diameter of tumor infiltration (7 of 10 cases, 70.0%) than in patients with 3/4, 2/4 and 1/4 diameter of tumor infiltration (3 of 25 cases, 12.0%; 3 of 45 cases, 6.7%; 1 of 16 cases, 6.3%) ($\chi^2 = 27.944$, $P = 0.000$). The lateral lymph node metastasis rate of poorly, moderately and well differentiated carcinoma was 30.0% (9 of 30 cases), 9.1% (4 of 44 cases) and 4.5% (1 of 22 cases), respectively ($\chi^2 = 8.569$, $P = 0.014$), indicating that tumor diameter ⩾ 5 cm, tumor infiltration and differentiation are risk factors for lateral lymph node metastasis of advanced low rectal cancer. Therefore, lateral pelvic lymphadenectomy should be performed following the management of patients with tumor diameter ⩾ 5 cm, tumor infiltration or differentiation.

It is well known that local recurrence is the most important prognostic factor for rectal carcinoma[29-31]. It was reported that local recurrence can be found in 4%-50% of patients with rectal carcinoma after curative resection, and lateral lymph node metastasis may be the important factor for local recurrence[15,16]. Ueno *et al*[15] reported that patients with lateral node metastases have an increased risk for local recurrence (44% *vs* 11.7%; $P < 0.001$) compared with those without lateral node metastases. Sugihara *et al*[16] also reported that positive lateral lymph nodes are significantly associated with increased local recurrence of rectal cancer. Similarly in this study, lateral lymph node metastasis was significantly correlated with local recurrence of advanced low rectal cancer. The local recurrence rate of advanced low rectal cancer was 64.3% (9 of 14 cases) and 11.0% (9 of 82 cases) in patients with and without lateral lymph node metastasis ($\chi^2 = 22.308$, $P = 0.000$), respectively, indicating that lateral pelvic lymphadenectomy can significantly reduce local recurrence of advanced low rectal cancer.

In the present study, patients without lateral lymph node metastasis had significant improvements in median survival (80.9 ± 2.1 m, 95% CI: 76.7-85.1 m *vs* 38 ± 6.7 m, 95% CI: 24.8-51.2 m) compared to those with lateral

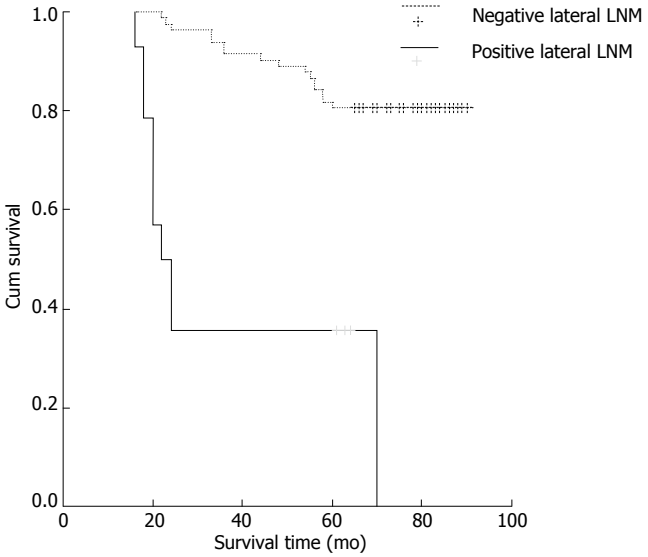

Figure 1 Relations between lateral lymph node metastasis and survival of patients with advanced low rectal cancer (Kaplan-Meier survival analysis). LNM: Lymph node metastasis.

lymph node metastasis. The difference between the two groups was statistically significant (log-rank, $P = 0.0001$), supporting that lateral lymph node metastasis has a significant prognostic value for advanced low rectal cancer. Lateral pelvic lymphadenectomy may effectively improve the survival of patients with advanced low rectal cancer. Ueno *et al*[32] also reported that advanced low rectal cancer patients having lymph node involvement in the lateral pelvic area are likely to benefit from lymphadenectomy.

COMMENTS

Background

Even having undergone radical resection with total mesorectal excision, about 5%-40% of patients with rectal cancer have local recurrence. The survival of patients with advanced low rectal cancer remains poor. Whether patients with advanced low rectal cancer could benefit from lateral lymph node dissection is still controversial.

Research frontiers

At present, lateral lymphadenectomy for advanced low rectal cancer is controversial. However, lateral lymph node metastasis is significantly associated with local recurrence and poor prognosis of advanced low rectal cancer.

Innovations and breakthroughs

The results of this study indicate that tumor diameter $\geqslant$ 5 cm, tumor infiltration and differentiation are risk factors for lateral lymph node metastasis of advanced low rectal cancer. Lateral lymph node metastasis is significantly correlated with local recurrence and prognosis of advanced low rectal cancer.

Applications

Lateral pelvic lymphadenectomy may effectively reduce local recurrence and improve the survival in patients with advanced low rectal cancer.

Peer review

The present study investigated the relations of lateral lymph node metastasis with local recurrence and survival in patients with advanced low rectal cancer. The study design is good and data analysis is extensive. The manuscript is well written.

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RAPID COMMUNICATION

Effect of Bcl-2 and Bax on survival of side population cells from hepatocellular carcinoma cells

Jing Fan, Ren Li, Rui Zhang, Hai-Liang Liu, Ning Zhang, Fu-Qing Zhang, Ke-Feng Dou

Jing Fan, Ren Li, Hai-Liang Liu, Ning Zhang, Fu-Qing Zhang, Ke-Feng Dou, Department of Hepatobiliary Surgery, Xijing Hospital, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Rui Zhang, Department of Biochemistry and Molecular Biology, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Correspondence to: Ke-Feng Dou, Department of Hepatobiliary Surgery, Xijing Hospital, the Fourth Military Medical University, 17 Changle Western Road, Xi'an 710032, Shaanxi Province, China. doukef@fmmu.edu.cn
Telephone: +86-29-84775255 Fax: +86-29-84775255
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Abstract

AIM: To understand the role and significance of side population (SP) cells from hepatocellular carcinoma (HCC) in hepatocarcinogenesis, development, relapse and metastasis, we simulated the denutrition conditions that cancer cells experience in clinical therapy, observed the different anti-apoptosis ability of SP cells and non-SP cells under such conditions, and established the possible effects of P53, Bcl-2 and Bax on survival of SP cells.

METHODS: We used flow cytometry to analyze and sort the SP and non-SP cells in established HCC lines MHCC97 and hHCC. We evaluated cell proliferation by methyl thiazolyl tetrazolium (MTT) assay and investigated the expression of p53, bcl-2 and bax genes during denutrition, by RT-PCR and immunofluorescence staining.

RESULTS: The percentage of SP cells in the two established HCC lines was 0.25% and 0.5%, respectively. SP cells had greater anti-apoptosis and proliferation ability than non-SP cells. Expression of Bcl-2 and Bax in SP and non-SP cells differed during denutrition. The former was up-regulated in SP cells, and the latter was up-regulated in non-SP cells.

CONCLUSION: It may be that different upstream molecules acted and led to different expression levels of Bcl-2 and Bax in these two cell lines. There was a direct relationship between up-regulation of Bcl-2 and down-regulation of Bax and higher anti-apoptosis ability in SP cells. It may be that the existence and activity of SP cells are partly responsible for some of the clinical phenomena which are seen in HCC, such as relapse or metastasis. Further research on SP cells may have potential applications in the field of anticancer therapy.

Key words: Side population; Hepatocellular carcinoma; Bax; Bcl-2; Apoptosis

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INTRODUCTION

It is believed that cancer is unicellular in origin[1], although cancer cells from a lot of tumors generally exhibit functional heterogeneity in experimental and clinical settings[2]. There are two theories[3,4]. One is the stochastic model, which figures that cancer is composed of a comparatively homogeneous population; only a few cells undergo stochastic events, so that they have the potential to proliferate extensively and form new tumors. The other is the hierarchy model, which suggests that there is some kind of pyramid scale in cancer cells. In this model, the subpopulation cells, which are on acme in the pyramid scale, comprise cancer stem cells (CSCs) that self-renew, generate downstream descendants, and initiate new tumors.

Recently, the latter hypothesis has gained significant recognition. The possible existence of CSCs has been shown in leukemia and some solid tumors, including breast cancer and brain tumors[5-9]. These cells are detected by their own ability to efflux Hoechst 33342 dye through an ATP-binding cassette (ABC) membrane transporter. They are also named side population (SP) cells for their location on flow cytometry charts.

Hepatocellular carcinoma (HCC) is a common malignancy and still has a high mortality rate[10]. Clinical operations and chemotherapy can lead most such cancer cells to death or proliferation inhibition through denutrition. However, there are always some cells that can survive and result in relapse or metastasis, which often leads to therapeutic failure and poor prognosis. The CSC hypothesis offers an explanation for these clinical phenomena. In some studies, it has been found that SP cells are easier to initiate tumors than non-SP cells are in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice[11]. Under conditions of denutrition

that are similar to those after clinical treatment, do SP cells survive more easily and have higher anti-apoptosis ability than non-SP cells?

p53, the most frequently mutated gene in human malignancies, is found inactivated in about 50% of tumors in any location and of any histological type. It has been named the "guardian of the genome"[12]. bcl-2 was the first example of an oncogene that inhibits cell death rather than promotes proliferation. According to differences in structure, the Bcl-2 family can be divided into two subgroups. Bax belongs to one subfamily that can oligomerize and integrate into the outer mitochondrial membrane to initiate events during apoptosis. Normally, Bcl-2 inhibits Bax activation. But, when cells are under stress (either oncogenic or genotoxic stress), Bcl-2 is inactivated by P53 as its downstream molecule. As a consequence, apoptotic cell death continues[13,14]. Under denutrition conditions, how are these three genes expressed in SP cells? What is their relationship with the anti-apoptosis ability of SP cells?

Taking clinical and experimental data together, we consider that the understanding of biologic characteristics of SP cells from HCC cells may serve to elucidate the mechanism of hepatocarcinogenesis and lead to novel therapeutic approaches.

In this study we therefore analyzed and sorted SP cells from established HCC cell lines. We observed the anti-apoptosis ability of two cell lines under denutrition conditions by methyl thiazolyl tetrazolium (MTT) assay. We further estimated the expression level of P53, Bcl-2 and Bax in SP cells and non-SP cells by RT-PCR and immunofluorescence during denutrition.

MATERIALS AND METHODS

Cell culture

The human liver cancer cell line MHCC97 was obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China), the hHCC cell line was from the Department of Biochemistry and Molecular Biology of Fourth Military Medical University (Xi'an, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen), and incubated at 37℃ in an atmosphere containing 5% CO_2.

SP cell analysis and purification using flow cytometry

Cells were detached from the dishes with trypsin-EDTA (Invitrogen) and suspended at 1×10^6 cells/mL in Hank's balanced salt solution (HBSS) supplemented with 3% fetal calf serum and 10 mmol/L HEPES. These cells were then incubated at 37℃ for 90 min with 20 μg/mL Hoechst 33342 (Sigma, St Louis, MO, USA), either alone or in the presence of 50 μmol/L verapamil (Sigma), which is an inhibitor of verapamil-sensitive ABC transporter. After 90 min incubation, the cells were centrifuged immediately for 5 min at 300 × *g*, 4℃ and resuspended in ice-cold HBSS. The cells were kept on ice to inhibit efflux of Hoechst dye. Then, 1 μg/mL propidium iodide (PI; BD Pharmingen, San Diego, CA, USA) was added to discriminate dead cells. Finally, these cells were filtered through a 40-μm cell strainer (BD Falcon; BD Pharmingen, San Diego, CA, USA) to obtain single suspension cells. Cell dual-wavelength analysis and purification were performed using dual-laser cytometry (FACSVantage; BD Biosciences, Franklin Lakes, NJ. Hoechst 33342 solution was excited at 355 nm UV light; blue fluorescence was collected with a 450/20 band-pass (BP) filter and red fluorescence with a 675-nm edge filter long-pass (EFLP). A 610-nm dichroic mirror short-pass was used to separate the emission wavelengths. PI-positive dead cells were excluded from the analysis.

MTT assay

All cells were maintained in PBS for 3, 6 and 9 h to induce denutrition. Then, cell proliferation was evaluated by MTT assay. MTT solution in PBS was added to a final concentration of 0.5 mg/mL, and cells incubated for 4 h at 37℃. Supernatant was removed and cells were resuspended in 150 μL DMSO for 10 min, and absorbance was measured at 490 nm using a microplate reader (Bio-rad, Japan).

Semi-quantitative RT-PCR

After all cells were induced in PBS for their respective number of hours, total RNA was extracted from SP and non-SP cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and was reverse-transcribed by using a First-Strand cDNA Synthesis Kit (Fermentas, Lithuania), as described in the instructions. RT-PCR was carried out in a 50 μL reaction mixture that contained 1 μL cDNA as template, 1 μmol/L specific oligonucleotide primer pair, and 25 μL Taq mixture that contained 0.5 U Taq DNA polymerase (Tangen, Beijing, China). Cycle parameters for p53, bcl-2, bax and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNAs were 15 s at 95℃, 30 s at 55℃, and 60 s at 72℃ for 33, 32, 30 and 20 cycles, respectively. Primer sequences were as follows: p53, 5'-GTTTCCGTCT GGGCTTCTTG-3' and 5'-CCTGGGCATCCTTGA GTTCC-3'; bcl-2, 5'-ACACTGTTAAGCATGTGCC G-3' and 5'-CCAGCTCATCTCACCTCACA-3'; bax, 5'-GGATGCGTCCACCAAGAA-3' and 5'-ACTCCCG CCACAAAGATG-3'; and G3PDH, 5'-ACCACAGTCC ATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCT GTA-3'.

Immunofluorescence staining

Cells were cultured on coverslips and received the same treatment of induced denutrition in PBS as described above. Cells were fixed in methanol at -20℃ for 20 min and washed in PBS containing 0.1% Tween 20 (Sanland Chemicals, Xiamen, China). After blocking with 10% goat normal serum for 1 h, fixed cells were incubated with primary antibodies, rabbit anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-Bax (Santa Cruz Biotechnology), in a moist chamber at 4℃ overnight. Cells were washed in PBS containing 0.1% Tween 20, blocked again for 30 min, and treated

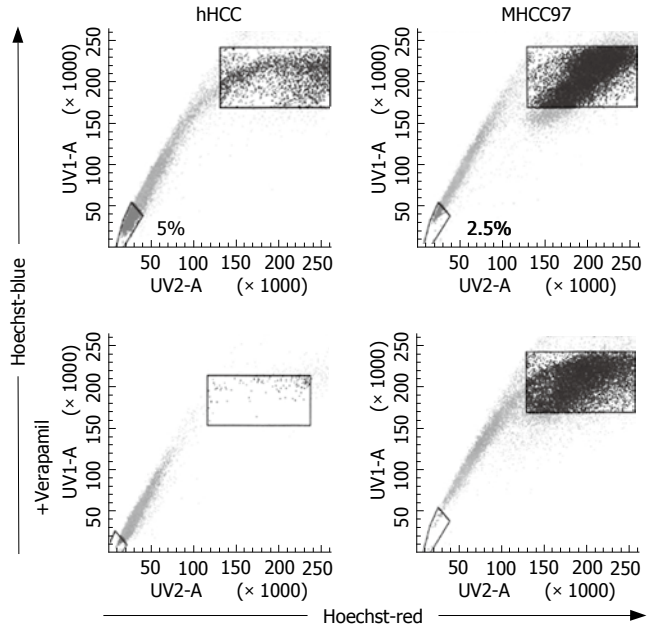

Figure 1 SP cells were detected in 0.25 and 0.5% of MHCC97 and hHCC cells, respectively. The SP cells disappeared with Hoechst 33342 and verapamil co-treatment.

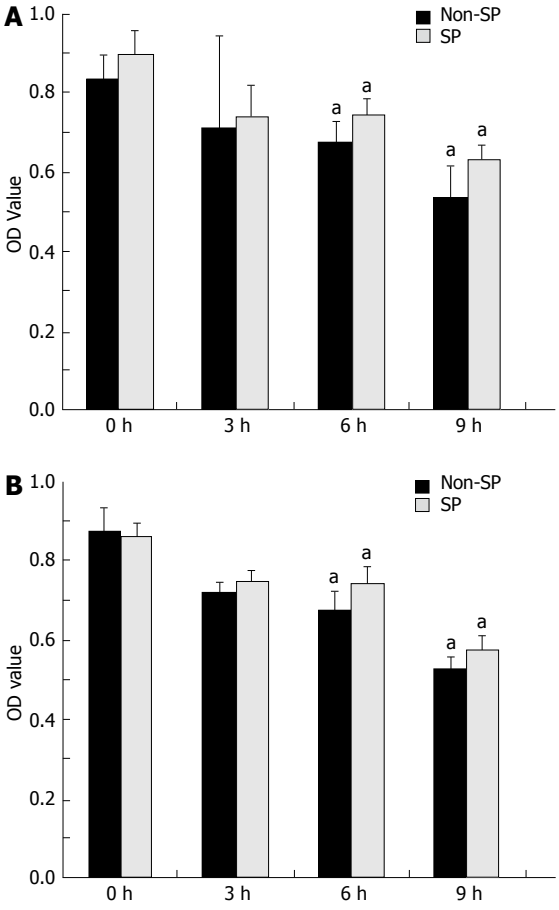

Figure 2 Proliferation of MHCC97 and hHCC cells at 3, 6 and 9 h during denutrition, as observed by MTT assay. SP cells purified from MHCC97 (**A**) and hHCC (**B**) cell lines demonstrated greater viability than the corresponding non-SP cells. $^{a}P < 0.05$.

with TRITC (Tetramethyl Rhodamine Isothiocyanate)-conjugated goat anti-rabbit IgG (Santa Cruz) and FITC (Fluorescein Isothiocyanate)-conjugated goat anti-mouse IgG (Santa Cruz) at 37℃ for 1 h. After washing in PBS, the coverslips were covered on slips with 30% glycerol phosphate buffer and examined under an Olympus IX70 microscope (Olympus, Japan).

Statistical analysis

Bands from RT-PCR were quantified by Smart View Bio-electrophoresis Image Analysis System software (Furi Science & Technology, Shanghai, China). Relative mRNA levels were calculated by referring them to the amount of G3PDH. Numerical data from the MTT assay were presented as the mean ± SEM. The difference between means was measured with Student's *t* test. All statistical analyses were performed using SPSS11.0 software (Chicago, IL, USA). $P < 0.05$ was considered as statistically significant.

RESULTS

Detection of SPs in HCC cells

Flow cytometry analysis with Hoechst 33342 staining demonstrated that MHCC97 and hHCC cells included 0.25% and 0.5% SP cells, respectively. The number of these SP cells was diminished in the presence of Hoechst 33342 and verapamil, a calcium channel blocker. The SP and non-SP cells in MHCC97 and hHCC cells were sorted separately and used for further experiments (Figure 1).

MTT assay

MTT assay was used to determine the proliferation of SP and non-SP cells under denutrition conditions. The results illustrated that denutrition inhibited proliferation of SP and non-SP cells in both cell lines in a time-dependent manner. The data showed that there was a difference between SP and non-SP cells at 6 and 9 h, and it seemed that SP cells had a greater proliferation ability than non-SP cells under denutrition (Figure 2).

RT-PCR

RT-PCR was used to detect mRNA expression levels in SP and non-SP cells in two cancer cell lines. The p53 gene was expressed weakly but steadily during the whole experiment. The bcl-2 gene was up-regulated, while the bax gene was down-regulated in SP cells under denutrition. Interestingly, the regulation of the two genes was reversed in non-SP cells. It may be that the upstream molecule which adjusted these two genes' expression was not P53 (Figure 3).

Immunofluorescence staining

Expression levels of Bcl-2 and Bax proteins were examined by immunofluorescence staining in hHCC and MHCC97 cell lines (Figure 4). It was clear that the expression of Bcl-2 increased in a time-dependent manner in SP cells, in contrast with non-SP cells. Conversely, the expression of Bax increased in non-SP cells in the same manner and decreased in SP cells.

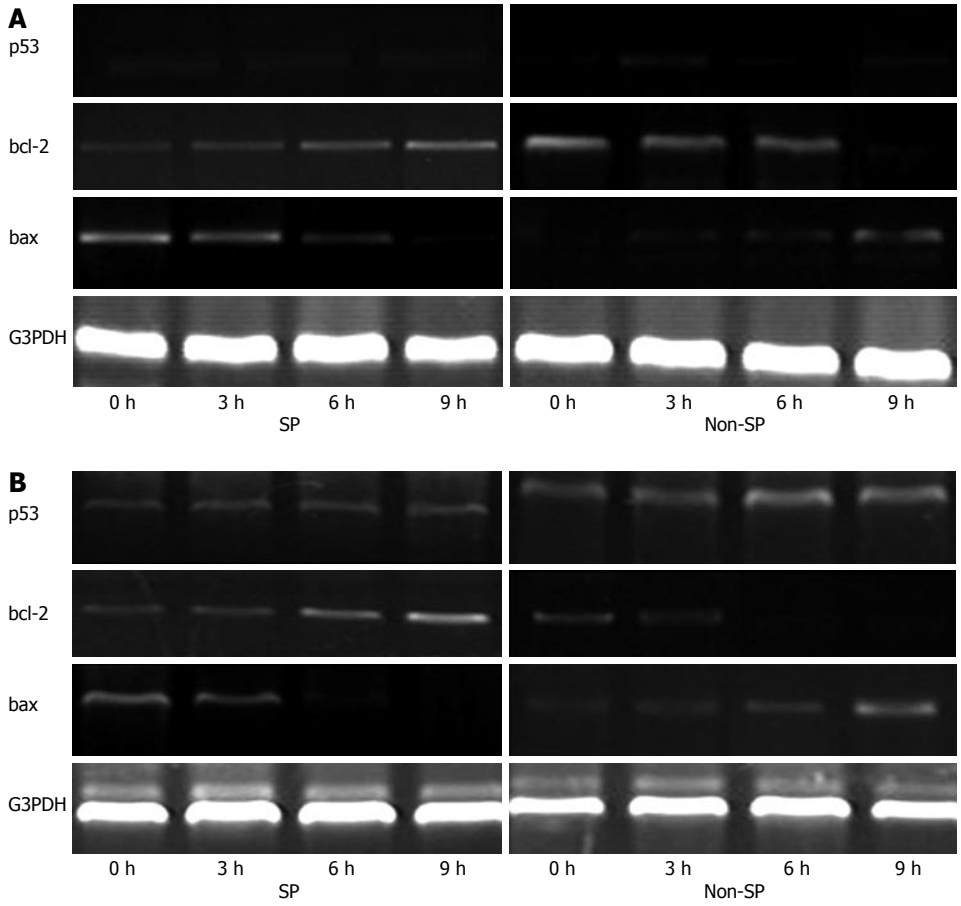

Figure 3 p53, bcl-2 and bax mRNA levels were evaluated by RT-PCR. bcl-2 levels were up-regulated in SP cells from MHCC97 (**A**) and hHCC (**B**) cell lines, and bax levels were down-regulated in non-SP cells during denutrition.

DISCUSSION

HCC ranks among the most common cancers in many countries. A recent estimate indicates that HCC represents the fifth most common cancer in males, and the eighth most common in females, with a total of 560 000 new cases each year, 83% of which occur in developing countries, and more than one-half in China alone. Moreover, because of its very poor prognosis, HCC represents the third leading cause of cancer death worldwide[10].

From the clinical standpoint, there are some conspicuous biological characteristics in HCC, such as anti-apoptosis, chemotherapy resistance, extensive proliferation, and even early metastasis. These characteristics are particularly prevalent in cases of relapse and metastasis.

Recently, it has been reported that CSCs are seen in many kinds of tumors and established cancer cell lines[15-18]. Using the previously described methods, we analyzed and sorted some of these CSCs, namely SP cells, in established HCC cell lines. The proportion of SP cells in the two cell lines was 0.25% and 0.5%, respectively. The MHCC97 (Metastatic Human Hepatocellular cancer 97) cell line was established from a highly metastatic case of HCC in 1997, and the hHCC (human Hepatocellular cancer) cell line was cultured from a case with a high level of chemotherapy resistance, with the proportion of SP cells being a little higher than those reported previously.

In clinic, whatever surgery or chemotherapy, it will bring denutrition directly or indirectly so as to inhibit the proliferation of cancer cells, beside destroy the structure of tumors or kill them. In clinics, surgery, radiotherapy and chemotherapy are used to destroy the structure of tumors, induce denutrition, kill cancer cells directly, and inhibit cancer cell proliferation. However, there are still some cells that can survive in denutrition conditions, and these may lead to relapse and metastasis. What difference is there between these and other cells? What mechanism is behind these phenomena? In our experiment, we simulated the denutrition conditions and observed the anti-apoptosis or proliferation ability of SP cells.

By MTT assay, we found that SP cells had better resistance to denutrition than non-SP cells. Using RT-PCR and immunofluorescence staining, we found that P53 may not be the key molecule that is responsible for the anti-apoptosis ability of SP cells. p53 was one of the most important genes in stabilizing the cell genome. It regulated the expression of numerous pro-apoptotic genes, such as bcl-2 and bax. Our results showed that the normal activity of P53 in the two cell lines may have been inhibited. The expression levels of two members of the Bcl-2 family were clearly altered between SP and non-SP cells; specifically, the expression of Bax was inhibited in SP and activated in non-SP cells, but the expression of Bcl-2 was reversed. Bax was a cytosolic monomer in viable cells but during apoptosis, it changed its conformation, integrated into the outer mitochondrial membrane, and was oligomerized. It provoked the permeabilization of the outer mitochondrial membrane (PT) and contributed to the release of pro-apoptotic factors into the cytosol, such as cytochrome C, which led to formation of the apoptosome and activation of the caspase cascade. However, the anti-apoptotic guardian Bcl-2 could bind Bax strongly, and this interaction

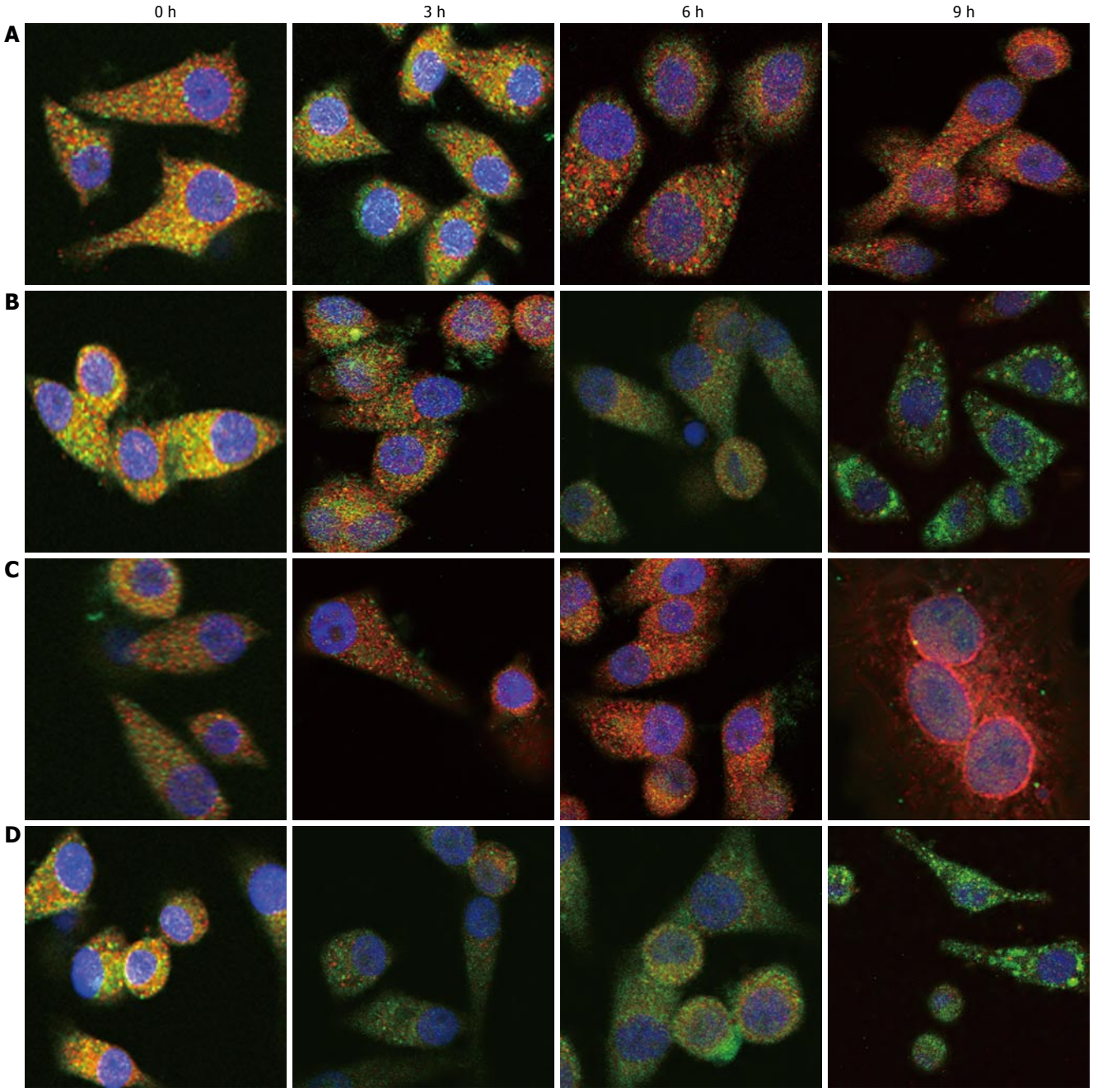

Figure 4 Bcl-2 (red) and Bax (green) expression was examined in SP (**A**) and non-SP (**B**) cells of MHCC97, and SP (**C**) and non-SP (**D**) cells of hHCC cells. The expression of Bcl-2 in SP cells was up-regulated, while Bax was down-regulated.

inhibited Bcl-2 activation, which sustained cell survival. In our study, the up-regulation of Bcl-2 and down-regulation of Bax were effective during the anti-apoptosis in SP cells. In other words, in MHCC97 and hHCC cell lines, SP cells had greater anti-apoptosis or proliferation ability than non-SP cells had. Expression of Bcl-2 and Bax had a pivotal role in the anti-apoptosis procedure during denutrition.

We have previously found that the expression level of alpha-fetoprotein (AFP) in SP cells is significantly higher than in non-SP cells in established HCC cell lines, e.g. MHCC97[19]. AFP is one of the most useful markers, and has been used in clinical diagnosis of HCC. AFP is synthesized in large quantities by the fetal yolk sac and the liver during embryonic development[20,21]. According to clinical experience, if a high level of AFP is found in the serum, the first thought is that the patient has HCC. If this appears after surgery or chemotherapy, it indicates a poor prognosis, such as recurrence or metastasis[22-27]. The ABC transporter for discharging Hoechst 33 342, which is called the breast cancer resistance protein, has a high efflux capacity with a wide substrate range, including mitoxantrone and methotrexate[28]. Further, the higher expression level of ABC transporter on SP cells indicates a possible relationship between them and clinical chemotherapy resistance.

Taking the experimental results and clinical experiences together, we found that the characteristics displayed in SP cells, such as high expression level of AFP and Bcl-2 and

drug efflux capacity, are consistent with the characteristics displayed in tumors, such as high expression level of AFP, high anti-apoptosis ability and chemotherapy resistance. We conclude that perhaps the existence and activity of SP cells are responsible for these clinical phenomena shown in HCC; moreover, it is reasonable to recognize SP cells as CSCs.

ACKNOWLEDGMENTS

We thank Mr. Shi-Liang Ma from Peking University Health Science Center for his patience and dedication during our flow cytometry analysis. We also thank Professor Qing-Chuan Zhao, Professor Zhen-Shun Song and Professor Yong Chen from the Department of Hepatobiliary Surgery for their helpful discussion during the preparation of this paper.

COMMENTS

Background

The theory of CSCs is one of the most significant theory in tumor research. According to the theory, there is some kind of pyramid cell structure in tumor cells, and CSCs are at its apex. They have self-renewal and differentiation abilities, and other cells are their descendents. CSCs have many biological activities, such as anti-drug and greater ability to proliferate. CSC theory also explains some clinic phenomena such as chemotherapy resistance and relapse. Therefore, research on the characteristics of CSCs may be the foundation of future clinical therapy.

Research frontiers

In 1996, Goodell sorted a certain kind of cells from mouse whole bone cells by cytometry, and he found that those cells expressed different biological characteristics, such as higher proliferation and multi-differentiation. Many workers have reported the existence of such cells in different tissues, and they have been collectively named SP cells. SP cells from tumors or tumor cell lines have greater proliferation and tumor formation ability, they resist drugs, and they maintain themselves in whole cells at a rate of a few percent.

Innovations and breakthroughs

The observation of SP cells backs up the theory of CSCs, which was introduced many years ago. Using Hoechst 33342 dye, it is possible to sort the cells by cytometry. SP cells have been found in HCC, brain tumor, prostate cancer and leukemia. By analyzing their biological abilities, it seemed that side population cells existed between the tip and bottom of the pyramid cell structure, and that they had intersection with real CSCs. Thus, though we can not be certain that SP cells are CSCs, it seems that that SP cells have a typical CSC phenotype.

Applications

In clinical cases, there have heretofore been many failures caused by tumor chemotherapy resistance or metastisis. CSC theory and the discovery of SP cells bring new hope for tumor therapy in the future. How to locate these cells in tumors, how to remove them completely by surgery, and how to inhibit or kill them by chemotherapy, are just a few of the clinical questions that need to be answered. Even a small advancement in this research field may result in a new breakthrough in tumor therapy.

Terminology

SP cells were first discovered by Goodell when he analyzed mouse bone cells by cytometry using Hoechst 33342. They were named after their location in 2-D cytometry charts. They display low fluorescence, and are located at the edge of the chart, away from other cells.

Peer review

SP cells from tumors or tumor cell lines are one of the hottest topics in tumor research. This study confirmed their existence and numbers in two human HCC cell lines.

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RAPID COMMUNICATION

Effect of fluoxetine on depression-induced changes in the expression of vasoactive intestinal polypeptide and corticotrophin releasing factor in rat duodenum

Yong-Lan Huang, Jie-Ping Yu, Gao-Hua Wang, Zhen-Hua Chen, Qing Wang, Ling Xiao

Yong-Lan Huang, Gao-Hua Wang, Zhen-Hua Chen, Ling Xiao, Department of Mental Health Center, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
Jie-Ping Yu, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
Qing Wang, Department of Gastroenterology, Wuhan University Zhongnan Hospital, Wuhan 430071, Hubei Province, China
Supported by Department of Mental Health Center and Department Gastroenterology of Renmin Hospital of Wuhan University, Hubei Province, China
Correspondence to: Professor Jie-Ping Yu, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan 430060, Hubei Province, China. luxiong100@21cn.com
Telephone: +86-27-63389801 Fax: +86-27-88072022
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Abstract

AIM: To investigate changes in vasoactive intestinal polypeptide (VIP) and corticotrophin releasing factor (CRF) in the plasma and duodenum of chronic stress-induced depressed rats and the effects of fluoxetine hydrochloride (fluoxetine) treatment on depression-induced changes in VIP and CRF.

METHODS: A Sprague-Dawley rat model of chronic stress-induced depression was produced. Thirty experimental rats were randomly divided into the following groups: control group, saline-treated depressed group, and fluoxetine-treated depressed group. Open-field testing was performed to assess the rats' behavior. VIP and CRF levels in plasma were measured by ELISA. Immunofluorescence techniques combined with laser scanning confocal microscopy (LSCM) were used to investigate VIP and CRF expression in the duodenum.

RESULTS: The open-field behavior, both crossing and rearing, of depression model rats, decreased significantly compared with those of normal control rats over 5 min. Defecation times increased significantly. Compared to the control group, FITC fluorescence of duodenal CRF expression and plasma CRF levels in the depressed rats increased significantly (fluorescence intensity of duodenal CRF: 11.82 ± 2.54 *vs* 25.17 ± 4.63; plasma CRF: 11.82 ± 2.54 ng/L *vs* 25.17 ± 4.63 ng/L, $P < 0.01$), whereas duodenal VIP expression and plasma VIP levels decreased significantly (fluorescence intensity of duodenal VIP: 67.37 ± 18.90 *vs* 44.51 ± 16.37; plasma VIP: 67.37 ± 18.90 ng/L *vs* 44.51 ± 16.37 ng/L, $P < 0.01$). Fluoxetine improved depressed behavior, increased VIP expression and decreased CRF expression in plasma and the duodenal tissue of depressed rats.

CONCLUSION: Chronic stress can induce injury to the duodenum, accompanied by increasing CRF and decreasing VIP in the plasma and duodenum. Treatment with fluoxetine can ameliorate pathological changes in the duodenum of depressed rats, which suggests that antidepressants are an effective therapeutic agent for some duodenal diseases caused by chronic stress. VIP is a potential therapeutic strategy.

Key words: Depression; Plasma; Duodenum; Rat; Vasoactive intestinal polypeptide; Corticotrophin releasing factor; Fluoxetine hydrochloride

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INTRODUCTION

In clinical studies, it has become clear that psychological factors, especially anxiety and depression, play an important role in gastrointestinal diseases by precipitating exacerbation of symptoms[1,2]. Several studies have shown that the prevalence of chronic stress disorders in patients with gastrointestinal symptoms is about 60%-85%[3,4]. Stress often worsens the symptoms of gastrointestinal diseases, which might be explained by altered neuroendocrine and visceral sensory responses to stress[5].

In recent years, along with extensive research on the enteric nerve system (ENS), increasing evidence shows that peptidergic neurotransmitters could regulate gastrointestinal diseases. Vasoactive intestinal peptide (VIP), a 28-amino acid peptide, was first discovered, isolated, and purified from porcine intestinal extracts[6]. It was also found in submucous and myenteric plexuses,

as well as the central and peripheral nervous systems[7]. It is now recognized as a major neuropeptide in the brain and gut, with functions ranging from neurotransmission to neuromodulation with neurotrophic properties. Corticotrophin releasing factor (CRF) is a 41 amino-acid peptide which stimulates adrenocorticotropic hormone (ACTH) secretion. Some data strongly suggest that CRF plays an important role in the pathophysiology of gastrointestinal diseases and electrophysiological properties of the brain during visceral perception[8]. Patients with gastrointestinal diseases may have a higher tone of corticotropin-releasing hormone (CRH) in the brain. In common, both central and peripheral nervous pathways are involved in the release of gastrointestinal hormones due to psychological stress, thus modulating gastrointestinal motility[9]. A large body of evidence derived from experiments suggests that CRF can accelerate small intestine transit, while VIP can inhibit it[10].

Fluoxetine is a SSRI (selective serotonin re-uptake inhibitor), which are a class of antidepressants used in the treatment of depression and anxiety disorders. SSRIs increase the extracellular expression of the neurotransmitter serotonin by inhibiting its re-uptake into the presynaptic cell. Serotonin is also involved in the regulation of carbohydrate metabolism. Few analyses of the role of SSRIs in treating depression have covered the effects on carbohydrate metabolism from intervening in serotonin handling by the body. Studies have suggested that SSRIs may promote the growth of new neural pathways or neurogenesis[11]. Also, SSRIs may protect against neurotoxicity caused by other compounds as well as from depression itself. Recent studies have shown that pro-inflammatory cytokine processes occur during depression in addition to somatic disease, and it is possible that symptoms manifested in these psychiatric illnesses are being attenuated by the pharmacological effects of antidepressants on the immune system[12]. SSRIs have been shown to be immunomodulatory and anti-inflammatory against pro-inflammatory cytokine processes[13,14].

However, there has been no report so far concerning the changes in VIP and CRF aroused by depression in plasma and duodenal tissue, and the effect of antidepressants on the duodenum. Therefore, we devised a rat depression model and observed the levels of VIP and CRF in the plasma and duodenum, and the effect of fluoxetine on the duodenum of depressed rats.

MATERIALS AND METHODS

Animals

Forty healthy male Sprague-Dawley rats, weighing 250 ± 30 g, from the Animal Center, Academy of Hubei Preventive Medical Sciences, were employed in the present study. The animals were fed standard rat chow, allowed access to tap water and were acclimated to their surroundings for 1 wk prior to the experiments. After this period, 30 rats were selected according to their open-field behavior.

Reagents

FITC (Fluorescein isothiocyanate)-conjugated goat anti-rabbit IgG, VIP and CRF rabbit anti- mouse antibodies were purchased from Sigma Co., USA. Fluoxetine hydrochloride capsules were purchased from Lilly Co. Ltd., and ELISA kits were purchased from Beijing SUNBIO Biological Technology Co. Ltd. Other reagents used in the study were all of analytical grade.

Experimental protocols (preparation of the rat depression model treated with saline or fluoxetine)

A rat model of chronic stress-induced depression was established[15-17]. The rats received a variety of stressors for 21 d, including tail nip for 1 min, cold water swimming at 4℃ for 5 min, heat stress at 45℃ for 5 min, water deprivation for 24 h, food deprivation for 24 h, 12-h inverted light/dark cycle (7:00 a.m. lights off, 7:00 p.m. lights on), paw electric shock (electric current 1.0 mA 10 s, every 1 min, lasting for 10 s, 30 times), *etc.* Stressors were administered throughout the experiment, could occur at any time of day (or night), and were each applied for a period of between 8 and 24 h. Their sequence was at random in order to be completely unpredictable to the animal. The animals were randomly divided into three groups (10 rats per group): model control, saline + chronic stress-induced, fluoxetine + chronic stress-induced therapy group. The depressed animals were treated with normal saline or fluoxetine (10 mg/kg) by stomach, (once a day, from the 24 h after the depressed model was established until the end of the experiment). A normal control group of rats (10 rats) without receiving any stress was included and housed in a separate room; food and water were freely available in their home cage.

Open field test (OFT)

The open-field test was designed to measure the reaction of rats to a novel environment. In this test, rats were individually placed in the center of a square, wooden, white-colored open-field box with 36 squares measuring 10 cm × 10 cm each. Their activity was assessed for 5 min. The number of squares from which rats crawled out was the total number of crossings. The number of occasions on which the animals stood on their hind legs was the total number of rearings. Defecation times were counted every 5 min. Each rat was housed in one cage and fasted before sucrose intake testing, after which 10 g/L sucrose solution consumption in 24 h was examined.

Assessment of duodenal histological damage

Duodenal tissue was sampled for a variety of determinations after the rats were anesthetized with 200 d/L urethane. Duodenal tissue was fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned in 4 μm thick sections, and stained with haematoxylin and eosin. The criteria of the histological score used was a previously validated scoring system from 0 to 4 that depends on the number and size of ulcers as well as the presence or absence of adhesions[18,19]: (1) the infiltration of acute inflammatory cells: 0 = no, 1 = mild increasing, 2 = severe increasing; (2) the infiltration of chronic inflammatory cells: 0 = no, 1 = mild increasing, 2 = severe increasing; (3) the deposition of fibrotin protein: 0 = negative, 1 = positive; (4) submucosa edema: 0 =

Table 1 Open-field activities and sucrose intake test of rats (n = 10, mean ± SD)

Group	Crossing/5 min	Rearing/5 min	Defecation/5 min	Sucrose intake mL/24 h
Control	133.00 ± 11.309	28.53 ± 10.22	3.90 ± 0.57	36.18 ± 10.24
Saline + depressed	51.80 ± 7.441[b]	11.10 ± 5.18[b]	6.80 ± 0.57[b]	8.95 ± 7.39[b]
FH + depressed	76.60 ± 3.534[d]	15.58 ± 7.367[d]	5.10 ± 0.43[d]	18.10 ± 6.43[d]

FH: Fluoxetine hydrochloride. [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* saline-treated depressed group.

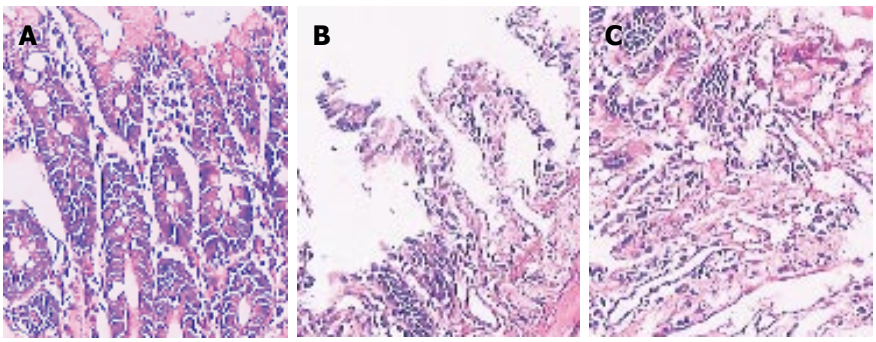

Figure 1 Haematoxylin and eosin staining of duodenum tissues. **A:** No damage in the normal control group (HE, × 200); **B:** Histological changes in the FH + depressed group (HE, × 200); **C:** Histological changes in the saline + depressed group (HE, × 200).

none, 1 = patchy-like, 2 = fusion-like; (5) epithelial necrosis: 0 = no, 1 = limiting, 2 = widening; (6) epithelial ulcers: 0 = negative, 1 = positive. The ulceration, inflammation, lesion and fibrosis were scored and put together as a result ranging between the minimum of 0 and maximum of 10.

Measurement of plasma VIP and CRF

Immediately after the rats were sacrificed, blood samples were collected into chilled tubes containing 0.3 μL ethylenediamine tetraacetic acid (EDTA) and 1000 KIU aprotinin. Blood samples were immediately centrifuged at 3500 r/min at 4℃ for 10 min. The supernatant was aspirated and stored at -70℃ until analysis. VIP and CRF were determined by enzyme linked immunosorbent assays (ELISA) according to the manufacturer's instructions.

Detection of duodenal VIP, CRF expression

Duodenal tissue was fixed in 4% paraformaldehyde for 4 h. One hundred micron sections from the primary tissue were employed in the fluorescent immunohistochemical analysis, which used rabbit anti-rat VIP/CRF antibody, diluted 1:500 in phosphate-buffered saline (PBS). The staining procedure was as follows: (1) the sections were washed in PBS, then pretreated with 0.25% Triton X-100 for 30 min at 37℃ and rinsed in PBS; (2) incubation for 12 h at 4℃ in a 1:500 dilution of the primary antibody of VIP /CRF in PBS; (3) incubation with 1:100 diluted secondary antibodies (FITC -conjugated goat anti-rabbit IgG) in PBS for 30 min at 37℃. The sections were washed three times for 10 min after incubation steps 1 to 3, respectively, and were finally mounted in 50 g/L glycerin.

Detection was carried out according to the kit instructions (Leica SP2 TCS AOBS made in Germany). The specimens were excited with a laser beam at a wavelength of 488 nm (FITC). Five visual fields in three sections of each tissue were randomly selected and observed under a laser scanning confocal microscope (LSCM) and analyzed with a Leica Q500IW image analysis system in terms of FITC fluorescent intensity. This study recorded the relative value of fluorescence intensity for the expression of VIP and CRF.

Statistical analysis

The data were expressed as the mean ± SD and analyzed with SPSS 11.5 statistic software. Statistical analysis was performed by using one-way ANOVA and Student-Newman-Keuls test for multiple comparisons. A P value less than 0.05 was considered statistically significant.

RESULTS

Open-field behavior of depression model rats (both crossing and rearing), was significantly decreased compared with that of the normal control rats (Table 1, $P < 0.01$). Defecation times significantly increased. The consumption of 10 g/L sucrose solution significantly decreased compared with that of the normal control (Table 1, $P < 0.01$). Treatment with fluoxetine hydrochloride (FH) significantly attenuated these effects.

Histological evaluation of the duodenum

No histological damage was seen in the normal control group. Rats with chronic stress-induced duodenitis showed neutrophil, macrophage, lymphocyte and eosinophil infiltration in the mucosa and submucosa. Ulceration and mucosal damage was obvious. Treatment with fluoxetine significantly attenuated the extent and severity of the histological signs. Damage scores of duodenum tissues were as follows: control: 0.39 ± 0.51; saline plus depressed: 7.46 ± 2.14; and FH plus depressed: 4.81 ± 1.37 (Figure 1).

VIP and CRF concentrations in plasma

Plasma VIP levels showed a significant difference among the three groups (Table 2, $P < 0.01$). VIP levels were higher in control and fluoxetine plus depression groups; however, they decreased significantly in the depressed group.

Plasma CRF levels showed a significant difference

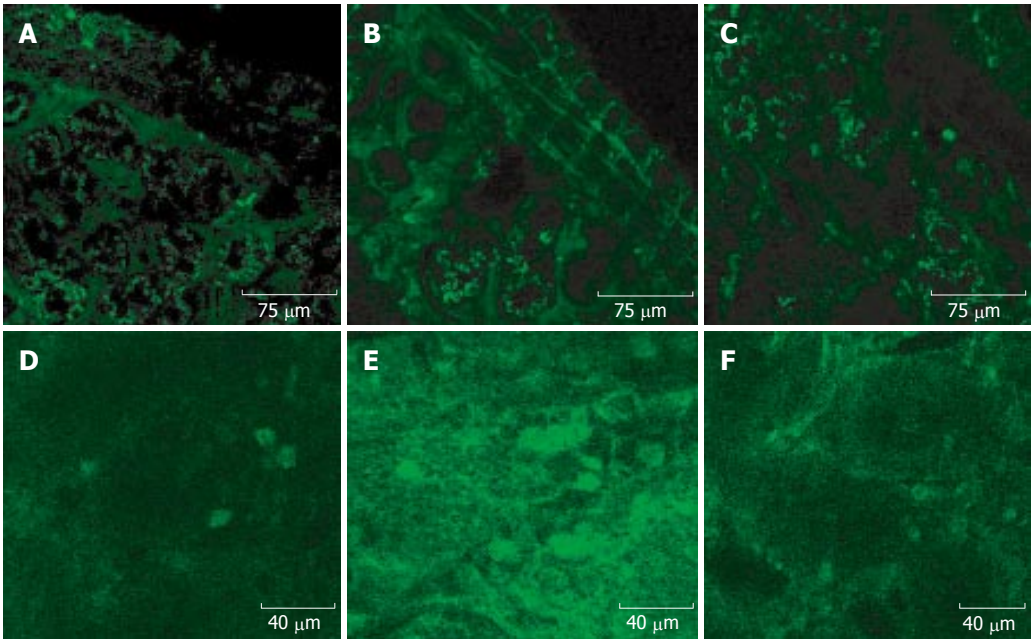

Figure 2 Duodenal tissue staining procedure included: (1) pretreatment with 0.25% Triton X-100; (2) incubation in the primary rabbit anti-rat antibody of VIP/CRF; (3) incubation with secondary antibodies (FITC -conjugated goat anti-rabbit IgG). Expression of FITC-labeled VIP/CRF (green). **A:** Expression of VIP in the control group; **B:** Expression of VIP in the depressed group; **C:** Expression of VIP in the FH + depressed group; **D:** Expression of CRF in the control group; **E:** Expression of CRF in the depressed group; **F:** Expression of CRF in the FH + depressed group.

Table 2 Changes of levels of VIP/CRF in plasma (n = 10, mean ± SD)

Group	VIP in Plasma (ng/L)	CRF in Plasma (ng/L)
Control	67.37 ± 18.90	11.82 ± 2.54
Saline + depressed	44.51 ± 16.37[b]	25.17 ± 4.63[b]
FH + depressed	60.86 ± 19.27[d]	17.05 ± 3.69[d]

[b]P < 0.01 *vs* control group; [d]P < 0.01 *vs* saline-treated depressed group.

among the three groups (Table 2, P < 0.01). CRF levels were lower in control and fluoxetine plus depression groups, but increased significantly in the depressed group.

VIP and CRF alterations and the effects of fluoxetine on the content of VIP and CRF in duodenum tissue of depression model rats

Compared with that of normal control rats, the average fluorescence intensity of duodenal CRF increased significantly, while the average fluorescence intensity of duodenal VIP decreased significantly in chronic stress-induced depressed rats (Table 3, P < 0.01). Furthermore, a significant improvement of the elevated duodenal VIP content and the significant reduction of duodenal CRF content were observed in animals treated with fluoxetine (Figure 2, Table 3, P < 0.01).

DISCUSSION

We found that experimental rats had almost all demonstrable symptoms of depression, consistent with the classic and mature model of depression[16,20]. Our results showed that both crossing and rearing behavior of depressed rats over five minutes significantly decreased compared with that of the normal control rats (Table 1, P < 0.01). Defecation times significantly increased. The consumption of 10 g/L sucrose solution significantly decreased compared with that of the normal control.

Table 3 The average fluorescence intensity analysis of VIP and CRF alterations in duodenum of depression model rats (n = 10, mean ± SD)

Group	Fluorescence intensity of VIP	Fluorescence intensity of CRF
Control	36.28 ± 17.16	10.87 ± 9.28
Saline + depressed	19.07 ± 13.84[b]	50.83 ± 24.66[b]
FH + depressed	28.29 ± 15.02[d]	29.18 ± 17.34[d]

FH: Fluoxetine hydrochloride; VIP: Vasoactive intestinal polypeptide; CRF: Corticotrophin releasing factor. [b]P < 0.01 *vs* control group; [d]P < 0.01 *vs* saline-treated depressed group.

Crossing reflected the degree of animal activity, rearing reflected the degree of curiosity to the novel surroundings, defecation times responded to intestinal function, and sucrose intake tests reflected the animal's response to rewards[21,22]. The chronic stressors caused a generalized decrease in action and responsiveness to rewards, and a functional gastrointestinal disorder. The behavioral changes of the depressed rat were reversed by chronic treatment with fluoxetine hydrochloride (a type of antidepressant).

On the other hand, rats with chronic stress-induced depression showed significant histological damage from duodenitis. For example, a number of neutrophils, macrophages, lymphocytes and eosinophils were found in the mucosa and submucosa; ulceration and mucosal damage could also be observed. No change was seen in the normal control group, and treatment with fluoxetine hydrochloride significantly attenuated the extent and severity of the histological signs. The antidepressant fluoxetine hydrochloride inhibited the extent of inflammation, prevented mucosa injury, minimized the ulceration area, and alleviated the duodenitis seen in the depressed animals.

In this study, we also found that there were different changes in VIP and CRF in the depressed rats' duodenum and plasma. The average fluorescence intensity of duodenal CRF expression of the depression model rats

increased significantly compared with that of the normal control rats, whereas that of duodenal VIP expression decreased significantly.

Brain gut peptides (BGPs) are distributed extensively in the brain and the gastrointestinal tract. Studies have demonstrated that some BGPs including VIP and CRF participate in gastrointestinal motility, secretion and absorption[23]. Several investigations have found that basal CRF levels have increased significantly during stress in patients. Because the gut and the brain are highly integrated and communicate in a bidirectional fashion largely through the ANS and HPA axis, patients also responded with higher expression of ACTH during stress and had higher basal expression of noradrenalin than the normal group. Stress induced exaggeration of the neuroendocrine response and visceral perceptual alterations occur during and after stress by CRF[6]. On the other hand, some studies have indicated that VIP participated in the modulatory effect of drugs on gastrointestinal motility and played an important role in gastrointestinal disorders caused by psychological stress[24]. It had been demonstrated that stress-induced plasma VIP expression decreased gastrointestinal transit disorder beyond a certain intensity range of stress. VIP had potent protective activity against sepsis and increased the survival rate of septic animals[25]. In this study, the significant increase in the expression of CRF possibly suggests that depression could induce an inflammatory response of the duodenum by releasing CRF in rats. The effect of VIP on inflammatory cells could be an additional important mechanism of its potent protective activity on chronic stress-induced duodenitis. The results of our studies suggest that gastrointestinal motility disorders during psychological stress may be partially mediated by release of VIP and CRF.

Our results also show that an antidepressant plays an important role in decreasing symptoms in depressed rats. The behavioral changes of depressed rats were reversed by chronic treatment with fluoxetine. Treatment with fluoxetine significantly attenuated the extent and severity of the histological signs. Fluoxetine at the therapeutic dose of 10 mg/kg was effective in decreasing the expression of CRF and increasing the expression of VIP in the duodenum of depressed rats. Some data has shown that antidepressants may adjust other brain gut peptides or unknown factors, and thus ameliorate the damage of chronic stress-induced gastrointestinal disorders[26-28]. Future serotegenic antidepressants may be made to specifically target the immune system by either blocking the actions of pro-inflammatory cytokines or increasing the production of anti-inflammatory cytokines[29,30].

In summary, brain-gut interaction and psychological factors altered not only the pathology of brain tissue, but also duodenal tissue. The results of our study show that depression can induce injury to the duodenum accompanied by increasing CRF and decreasing VIP. Treatment with fluoxetine can ameliorate pathological changes in the duodenum in depressed rats, suggesting that SSRIs are an effective therapeutic agent for some duodenum diseases caused by psychological factors. We suggest that VIP prevention of inflammatory cell reactivity could be a potential therapeutic strategy for chronic stress-induced gastrointestinal disorders.

COMMENTS

Background

In clinical studies, it has become clear that depression plays an important role in gastrointestinal diseases by precipitating exacerbation of symptoms. Stress often worsens the symptoms of gastrointestinal diseases.

Research frontiers

Some data strongly suggested that corticotrophin releasing factor(CRF) and vasoactive intestinal peptide(VIP) played important roles in pathophysiology of gastrointestinal diseases. Selective serotonin reuptake inhibitors (SSRI's) have been shown to be immunomodulatory and anti-inflammatory against pro-inflammatory cytokine processes.

Innovations and breakthroughs

We set up a rat depression model, and observed the level of VIP and CRF of plasma and duodenum and the effect of fluoxetine on duodenum of the depressed rats.

Applications

The authors of the present study showed that chronic stress can induce injury to the duodenum. This was accompanied by an increase in immunofluorescence staining for CRF and a decrease in immunofluorescence staining for VIP. Treatment with the serotonin reuptake inhibitor, fluoxetine, reversed these changes and in addition reversed the behavioral changes of depressed rats.

Terminology

Vasoactive intestinal peptide (VIP) was found in submucous and myenteric plexus, central and peripheral nervous systems. It is now recognized as a major neuropeptide in the brain and gut. Corticotrophin releasing factor (CRF) stimulates adrenocorticotropic hormone (ACTH) secretion and plays an important role in the pathophysiology of gastrointestinal diseases.

Peer review

The authors of the present study showed that chronic stress can induce injury to the duodenum. This was accompanied by an increase in immunofluorescence staining for CRF and a decrease in immunofluorescence staining for VIP. Treatment with the serotonin reuptake inhibitor, fluoxetine, reversed these changes but in addition reversed the behavioral changes of depressed rats.

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RAPID COMMUNICATION

Protective effect of inducible nitric oxide synthase inhibitor on pancreas transplantation in rats

Bai-Feng Li, Yong-Feng Liu, Ying Cheng, Ke-Zhong Zhang, Tie-Min Li, Ning Zhao

Bai-Feng Li, Yong-Feng Liu, Ying Cheng, Ke-Zhong Zhang, Tie-Min Li, Ning Zhao, Department of Surgery and Organ Transplant Unit, The First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China
Supported by The Natural Science Foundation of Liaoning Province, No. 00225001
Correspondence to: Dr. Yong-Feng Liu, Department of Surgery and Organ Transplant Unit, The First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China. yfliu@mail.cmu.edu.cn
Telephone: +86-24-83283308
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Abstract

AIM: To investigate the effect of inducible nitric oxide synthase inhibitor, aminoguanidine, on pancreas transplantation in rats.

METHODS: A model of pancreas transplantation was established in rats. Streptozotocin-induced diabetic male Wistar rats were randomly assigned to sham-operation control group (n = 6), transplant control group (n = 6), and aminoguanidine (AG) treatment group (n = 18). In the AG group, aminoguanidine was added to intravascular infusion as the onset of reperfusion at the dose of 60 mg/kg, 80 mg/kg, 100 mg/kg body weight, respectively. Serum nitric oxide (NO) level, blood sugar and amylase activity were detected. Nitric oxide synthase (NOS) test kit was used to detect the pancreas cNOS and inducible NOS (iNOS) activity. Pancreas sections stained with HE and immunohistochemistry were evaluated under a light microscope.

RESULTS: As compared with the transplant control group, the serum NO level and amylase activity decreased obviously and the evidence for pancreas injury was much less in the AG group. The AG (80 mg/kg body weight) group showed the most significant difference in NO and amylase (NO: 66.0 ± 16.6 *vs* 192.3 ± 60.0, P < 0.01 and amylase: 1426 ± 177 *vs* 4477 ± 630, P < 0.01). The expression and activity of tissue iNOS, and blood sugar in the AG (80 mg/kg body weight) group were much lower than those in the transplant control group (iNOS: 2.01 ± 0.23 *vs* 26.59 ± 5.78, P < 0.01 and blood sugar: 14.2 ± 0.9 *vs* 16.8 ± 1.1, P < 0.01).

CONCLUSION: Selective iNOS inhibitor, aminoguanidine as a free radical, has a protective effect on pancreas transplantation in rats by inhibiting NO and reducing its toxicity.

INTRODUCTION

Pancreas transplantation is frequently complicated by acute pancreatitis, largely due to ischemia/reperfusion injury secondary to cold preservation[1,2]. During the reperfusion period, oxygen-derived free radicals can lead to a severe impairment. Nitric oxide (NO) is a free radical with a strong reactivity, and has a fierce cytotoxicity. However, NO can significantly dilate blood vessels and remit vasospasm of grafts. Therefore, NO plays an ambivalent role in ischemia/reperfusion during pancreas transplantation. In this study, we established a model of pancreas transplantation in rats to investigate the expression of nitric oxide synthase (NOS) isoforms, and the effect of inducible nitric oxide synthase (iNOS) inhibitor (aminoguanidine) on pancreas transplantation.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250-300 g (Experimental Animal Center, China Medical University, China) were used as donors and recipients. The animals were kept in standard conditions with free access to water and rodent chow. Diabetes was induced by intravenous injection of streptozotocin at a single dose of 55 mg/kg body weight. Only rats with non-fasting plasma glucose levels of more than 22 mmol/L were used as recipients. We performed recipient transplantation surgery on days 14 and 15 after the injection of streptozocin. A total of 30 recipient animals were randomly assigned to the sham-operation group (n = 6) in which animals underwent midline laparotomy only, transplant control group (n = 6) in which animals underwent transplantation and received a bolus injection of saline instead of aminoguanidine, and aminoguanidine treatment group (n = 18) in which animals

Table 1 Serum NO level and amylase activity 4 h after transplantation (mean ± SD)

Group	*n*	NO (μmol/L)	Amylase (U/dL)
Sham-operation group	6	30.0 ± 3.5	342 ± 73
Transplant control group	6	192.3 ± 60.0[b]	4477 ± 630[f]
AG-60 mg/kg body weight	6	137.3 ± 21.1	2848 ± 354
AG-80 mg/kg body weight	6	67.9 ± 19.5[d]	1494 ± 263[h]
AG-100 mg/kg body weight	6	66.0 ± 16.6	1426 ± 177

[b]$P < 0.01$, [f]$P < 0.01$ *vs* sham-operation group; [d]$P < 0.01$, [h]$P < 0.01$ *vs* transplant control group.

underwent transplantation. Before reperfusion, a bolus injection of aminoguanidine (60 mg/kg, 80 mg/kg or 100 mg/kg body weight) was given *via* the vena dorsalis penis.

Transplantation and collection of specimens

Synergetic pancreaticoduodenal transplantation was performed in diabetic recipients to assess islet cell functions. After overnight fasting with free access to water, the rats were anesthetized and underwent heterotopic pancreaticoduodenal transplantation as previously described[3] with certain modifications. After shaving and disinfecting the abdomen with 75% alcohol, a midline incision was made. The donor pancreas was isolated on an aortic segment branching off the celiac axis and the superior mesenteric artery. The venous outflow was provided by the portal vein. Pancreas grafts were flushed with and stored in cold (4℃) heparinized lactate Ringer's solution. Heterotopic intra-abdominal transplantation was performed by end-to-side anastomosis of the aortic segment of the graft and the recipient infrarenal aorta. The graft portal vein was anastomosed to the recipient vena cava using the same technique. Enteric diversion of exocrine graft secretion was accomplished by end-to-side duodenojejunostomy. The abdomen was closed in two layers with 2-0 silk suture. After a single intramuscular injection of 5 mg cefamandole post-operation, the rats were kept under warming lamps until they became active. The warming and cooling ischemic time was less than 15 min and 25 min, respectively. The animals were killed after 4 h of reperfusion. The pancreas was harvested and divided into two segments with one fixed in 10% PBS formalin and the other preserved at -70℃. The blood was withdrawn without anticoagulant and centrifuged at 2000 r/min for 10 min. The serum was preserved at -20℃.

Determination of serum NO and NOS levels

Nitrate reductase was used to detect the serum NO level and NOS test kit was used to detect the cNOS and iNOS activity in the pancreas.

Determination of serum blood and amylase levels

Serum glucose concentration was measured with an Exac Tech blood glucose meter in samples collected from the cut tip of the tail. Serum amylase concentration was measured with a multianalyzer (Clinilizer, CL-7150, Nippon Denshi, Tokyo, Japan).

Histopathology examination

One pancreas segment was fixed in 10% PBS formalin, dehydrated through a grade ethanol series, washed in xylene and embedded in paraffin. The segment was cut into 4 μm-thick sections. The sections were stained with haematoxylin and eosin and evaluated using light microscope.

Immunohistology

Primary antibody and anti-iNOS polyclonal antibody were produced in rabbits. Strept avidin-biotin complex immunoperoxidase staining system was used, and the positive staining was reddish-brown in color.

Statistical analysis

The data were presented as mean ± SD. All statistical analyses were performed using the SPSS 10.0 software. Differences in groups were tested by analysis of variance (ANVOA). $P < 0.05$ was considered statistically significant.

RESULTS

Serum NO level

The NO level increased significantly in transplant control group and deceased in the sham-operation group ($P < 0.01$) 4 h after reperfusion. After administration of aminoguanidine (AG), a selective iNOS inhibitor, NO level decreased significantly ($P < 0.01$). The effect of AG (80 mg/kg body weight) was obviously better than that of AG (60 mg/kg body weight) ($P < 0.01$). However, the effect of AG (100 mg/kg body weight) was not better than that of AG (80 mg/kg body weight) ($P > 0.05$) (Table 1).

Serum amylase activity

The amylase activity was higher in the transplant control group than in sham-operation group ($P < 0.01$). After administration of AG, the amylase activity decreased markedly, and the effect of AG (80 mg/kg body weight) was better ($P < 0.01$) (Table 1).

Blood sugar level

The blood sugar level decreased after pancreas transplantation, and was the lowest in the AG (80 mg/kg body weight) group ($P < 0.01$) (Table 2).

Activity of NOS isoforms in pancreas tissue

Four hours after reperfusion, the iNOS activity in pancreas tissue increased significantly ($P < 0.01$), but the cNOS activity had no change ($P > 0.05$). After administration of AG (80 mg/kg body weight), the iNOS activity decreased obviously ($P < 0.01$) while the cNOS activity remained normal (Table 3).

Histology

The pancreas was enlarged and swollen in the transplant control group, and appeared relatively normal in all animals of the sham-operation and AG (80 mg/kg body weight) groups. The microscopic pancreatic injury, as indicated by intracytoplasmic vacuoles, interstitial oedema, polymorphonuclear cell infiltrate, venous congestion, and

Table 2 Blood sugar level 4 h after transplantation (mean ± SD)

Group	*n*	Pretransplantation (mmol/L)	Posttransplantation (mmol/L)
Sham-operated control	6	19.6 ± 1.4[a]	-
Transplant control group	6	20.1 ± 2.0[a]	16.9 ± 2.0
AG-60 mg/kg body weight	6	19.9 ± 1.5[a]	16.8 ± 1.1
AG-80 mg/kg body weight	6	19.8 ± 1.7[a]	14.2 ± 0.9[b]
AG-100 mg/kg body weight	6	20.5 ± 1.6[a]	15.1 ± 1.8[c]

[a]$P > 0.05$, [b]$P < 0.01$ *vs* AG-60 mg/kg body weight post transplantation; [c]$P > 0.05$ *vs* AG-80 mg/kg body weight post transplantation.

Table 3 Activity of NOS isoforms in pancreatic tissue 4 h after transplantation (mean ± SD)

Group	*n*	cNOS (U/mL)	iNOS (U/mL)
Sham-operation group	6	5.35 ± 1.01[a]	1.87 ± 0.19
Transplant control group	6	5.91 ± 0.71[a]	26.59 ± 5.78[b]
(AG-80 mg/kg body weight)	6	5.64 ± 0.97[a]	2.01 ± 0.23[d]

[a]$P > 0.05$, [b]$P < 0.01$ *vs* sham-operation group; [d]$P < 0.01$ *vs* transplant control group.

local tissue hemorrhage and necrosis occurred 4 h after transplantation (Figure 1A and B). However, none of the samples from the AG (80 mg/kg body weight) group revealed histological evidence of pancreatic injury (Figure 1C and D).

Immunohistochemistry

Four hours after reperfusion, heavily stained specimens from transplant control group were positive for anti-iNOS, while iNOS staining was mainly localized on the endothelium, vascular smooth muscle, and islet cells (Figure 1E and F). No stained anti-iNOS antibody was detected in all specimens from the AG (80 mg/kg body weight) group (Figure 1G and H).

DISCUSSION

A model of pancreas transplantation in rats was established. Four hours after pancreas graft reperfusion, the expression and activity of iNOS on pancreas increased significantly, serum NO level and amylase activity, leading to severe pancreatitis, whereas cNOS remained normal. After administration of AG, the iNOS activity and NO concentration decreased, the toxicity of NO as free radicals was reduced, and the amylase activity decreased markedly. The severity of ischemia/reperfusion injury and postgraft pancreatitis was reduced, protecting the pancreas graft against ischemia/reperfusion injury.

Ischemia/reperfusion injury remains a major problem in pancreas transplantation. During the reperfusion period, endothelial dysfunction, activation of endogenous enzymes, leucocyte recruitment and activation all lead to generation of oxygen-derived free radicals, promote lipid peroxidation and deplete glutathione and other antioxidation compounds, leading to pancreatitis[4]. Contradictory results about the role of NO in pancreatic ischemia/reperfusion have been reported[5]. NO may lose an electron to form nitrosonium cation (NO^+), which can combine with the superoxide radicals to form peroxynitrite ($ONOO^-$), a highly active free radical with fierce cytotoxicity[5]. In pathological conditions, significant activation of iNOS by the release of inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), can increase NO concentration[6]. It was reported that endogenous NO is involved in formation of pancreatic edema in L-arginine-induced acute pancreatitis by increasing the vascular permeability and protein extravasation[7]. Treatment with L-NAME significantly reduces amylase activity and edema formation in the pancreas. A study about severe acute pancreatitis has shown a positive correlation between serum NO level and the number of adherent leucocytes[8]. The expression of iNOS is correlated to changes in the pancreatic histomorphology[9-12]. The expression of iNOS during reperfusion following pancreatic ischaemia contributes significantly to the development of acute pancreatitis[13]. Vasoactive mediators, such as bradykinin, platelet activating factor, endothelin and NO, participate in the development of pancreatic microcirculatory failure. Recently, in drug-induced pancreatitis models, some researchers found that there is a correlation among NF-kappaB activation, serum amylase, reactive oxygen species level and tissue damage, suggesting that NF-kappaB and iNOS play a key role in the pathogenesis of acute pancreatitis[14-16]. After treatment with antioxidants or NOS inhibitors, the levels of myeloperoxidase, serum amylase and NO, as well as iNOS activities are decreased significantly, and the pancreatic inflammation is improved[14,15]. Ma *et al*[16] found that the expression of NF-kappaB and iNOS in peritoneal macrophages is significantly higher in rats with severe acute pancreatitis, and anti-inflammatory agents decrease the expression of TNF-alpha, IL-1 and NO in peritoneal macrophages, reducing the severity of pancreatitis. In Folch-Puy's experiment, infusion of a contrast medium into the pancreatic duct could result in an inflammatory process characterized by increased lipase levels in plasma and edema as well as increased myeloperoxidase activity in pancreas, suggesting that activation of NF-kappaB is correlated with iNOS expression in pancreatic cells[17]. It was reported that ischemia/reperfusion provokes severe acute necrotizing pancreatitis with a high mortality rate and leads to systemic inflammatory reaction due to the activation of cytokine cascade and iNOS, indicating that NO overproduction by iNOS corresponds with the apoptotic process in the pancreas and the lung[18,19]. In a study on ischemia/reperfusion injury, Duchen found that calcium overload is associated with NO generation, and their combination leads

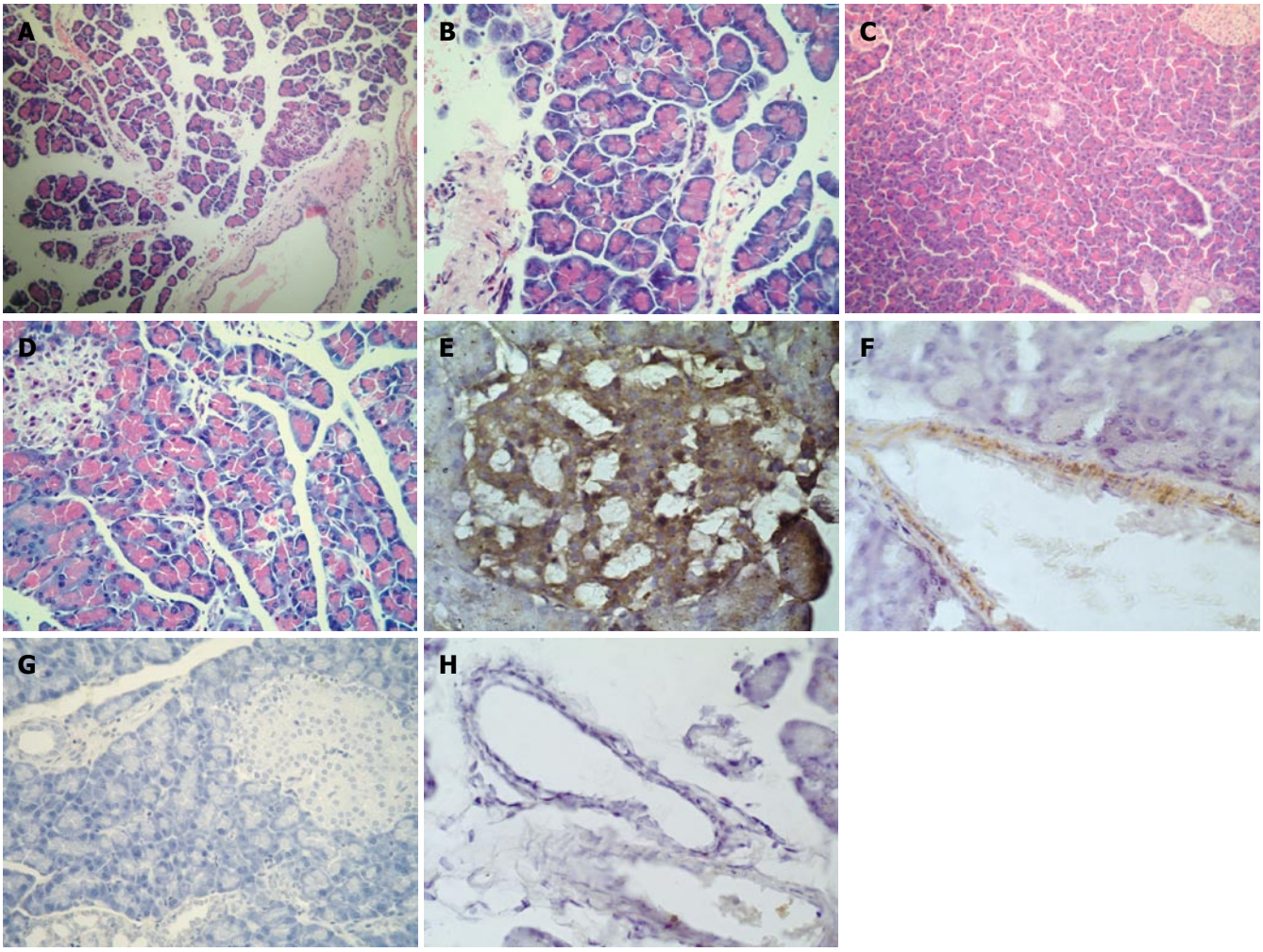

Figure 1 Histology displaying pancreatic injury (**A**, **B**) and no pancreatic injury (**C**, **D**), while immunohistochemistry showing positive anti-iNOS (**E**, **F**) and no stained anti-iNOS (**G**, **H**) in different groups.

to collapse of mitochondrial membrane potential followed by cell death[20]. It was reported that after administration of selective iNOS inhibitors, the iNOS activity and NO concentration are decreased significantly and the severity of pancreatitis is reduced[21]. However, some experiments indicate that NO could activate guanylate cyclase, reduce the activity of platelets and inflammatory cells, relax smooth muscle, and dilate blood vessels[22,23]. Therefore, NO can remit the vasospasm of grafts and decrease the occurrence of vascular crisis. Supplement of NO for donors during reperfusion of pancreatic isografts seems to prevent organ injury because NO attenuates leukocyte-dependent tissue injury[26]. Thus, it remains debatable whether the increased production of NO due to pancreas transplantation is beneficial or detrimental to the tissue.

Based on the findings of this study and present reports, it is very likely that NO plays a dual role in ischemia/reperfusion injury of pancreas. During the early reperfusion period, NO, under the charge of cNOS, can improve postischemic reperfusion. With the prolongation of reperfusion time, NO depletion results in failure of microcirculation, during which supplement of NO or NOS substrate could protect microcirculation against failure[24]. When reperfusion is prolonged (more than 4 h), activation and excessive expression of iNOS due to the release of inflammatory agents such as TNF-α, IL1-β, result in a considerable increase in NO concentration, and the toxic effect of NO as a free radical leads to the development of graft pancreatitis[25]. Therefore, administration of selective iNOS inhibitors can not only reduce the toxicity of NO as a free radical, but also retain vasodilatation effect, and protect the graft against pancreatitis. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of covalent modification of the iNOS protein and heme residue at the active site[26].

At present, some experiments demonstrated that in the transplanted islets, iNOS and toxic NO are produced due to infiltration of inflammatory cells into islets and production of proinflammatory cytokines (such as TNF-α, IL1-β), and an excessive production of NO is deleterious to pancreas β-cells[27-29].

In conclusion, selective iNOS inhibitor, aminoguanidine as a free radical, has a protective effect on pancreas transplantation in rats by inhibiting NO and reducing toxicity.

COMMENTS

Background

Pancreas transplantation is frequently complicated by acute pancreatitis, largely due to ischemia/reperfusion injury. During the reperfusion period, nitric oxide (NO) may form peroxynitrite ($ONOO^-$), a highly active free radical, and has a fierce cytotoxicity. However, NO can significantly dilate blood vessels and remit the vasospasm of grafts, protecting pancreas graft from thrombosis due to transplantation. Therefore, NO plays an ambivalent role in ischemia/reperfusion injury during pancreas transplantation. However, contradictory results about the role of NO in pancreatic ischemia/reperfusion injury have been reported. It remains debatable whether the increased production of NO due to pancreas transplantation is beneficial or detrimental to the tissue.

Research frontiers

Based on the findings of this study and recent reports, it is very likely that NO plays a dual role in ischemia/reperfusion injury of pancreas. During the early reperfusion period, NO under the charge of cNOS, could improve pancreas perfusion. With the prolongation of reperfusion time, NO depletion could result in failure of microcirculation, during which supplement of NO or NOS substrate can protect microcirculation against failure. When reperfusion is prolonged, activation of iNOS due to the release of inflammatory agents, such as tumor necrosis factor α and interleukin-1β, can increase NO concentration. The toxic effect of NO as a free radical can lead to graft pancreatitis. Hence, administration of selective iNOS inhibitors can reduce the toxicity of NO, and protect graft against pancreatitis.

Innovations and breakthroughs

We established a model of pancreas transplantation in rats. Administration of selective iNOS inhibitors could not only reduce the toxicity of NO as a free radical, but also retain vasodilatation effect, and protect graft against pancreatitis. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of the iNOS protein and heme residue at the active site. Our data also suggest that blood sugar level in AG group was much lower than that in transplant control group, indicating that the selective iNOS inhibitor, AG, has a protective effect on pancreas transplantation.

Applications

Pancreas transplantation can give IDDM additional pancreas to take the place of its own, which has lost the function of insulin secreting. Pancreas regulates insulin secretion, and maintains the blood glucose level. At present, nothing else could achieve this object. Factors influencing pancreas functions following transplantation include graft pancreatitis and rejection which are difficult to treat with a poor prognosis. In this study, after administration of selective iNOS inhibitor AG, the iNOS and amylase activity and NO concentration were decreased, the toxicity of NO as a free radical was reduced. The severity of ischemia/reperfusion injury and postgraft pancreatitis was reduced, protecting the graft against pancreastitis.

Terminology

Inducible nitric oxide synthase (iNOS): NO is synthesized from L-arginine by nitric oxide synthase (NOS). iNOS does not express at normal conditions, and produces NO several orders greater than cNOS and may have a more important pathological role. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of the iNOS protein and heme residue at the active site.

Peer review

This study investigated the effect of inducible nitric oxide synthase inhibitor, aminoguanidine, on pancreas transplantation, showing its scientific and clinical values.

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RAPID COMMUNICATION

Polypropylene mesh-reinforced pancreaticojejunostomy for periampullar neoplasm

Di-Yu Huang, Xian-Fa Wang, Wei Zhou, Ying Xin, Yi-Ping Mou, Xiu-Jun Cai

Di-Yu Huang, Xian-Fa Wang, Wei Zhou, Ying Xin, Yi-Ping Mou, Xiu-Jun Cai, Depatment of Surgery, Sir Run Run Shaw Hospital, School of Medicine of Zhejiang University, Institute of Microinvasive Surgery of Zhejiang University, Hangzhou 310016, Zhejiang Province, China
Correspondence to: Dr. Xian-Fa Wang, Depatment of Surgery, Sir Run Run Shaw Hospital, Medical School of Zhejiang University, Institute of Microinvasive Surgery of Zhejiang University, 3 East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. hdysrrsh@hotmail.com
Telephone: +86-13957119659 Fax: +86-571-86044817
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Abstract

AIM: To evaluate the effect of polypropylene mesh-reinforced pancreatojejunostomy on pancreatic leakage.

METHODS: Seventeen consecutive patients with paraampullar malignancy received polyprolene mesh-reinforced pancreatodudeonectomy and the Child's method was used to rebuild the alimentary tract.

RESULTS: The mean time of polyprolene mesh-reinforced pancreatojejunostomy was 22 min. Anastomosis could endure 30-500 cm H_2O pressure during operation. All patients recovered without pancreatic leakage.

CONCLUSION: Polyprolene mesh-reinforced pancreato-jejunostomy is a feasible and reliable procedure to prevent pancreatic leakage.

Key words: Pancreatic leakage; Pancreatojejunostomy; Anastomosis; Pancreatodudenectomy

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INTRODUCTION

Pancreatic anastomotic leakage is a common lethal complication of pancreaticojejunostomy. The incidence of pancreatic anastomosis leakage depends on multiple factors, among which the anastomotic method is the key factor. Different anastomotic methods in pancreaticojejunostomy have been reported in the literature, but none of them is able to prevent pancreatic leakage. Recently, we designed a new anastomotic method: polypropylene mesh-reinforced pancreaticojejunostomy (MRP), by which the sheath of jejunum is bound to the pancreatic remnant wrapped by a strip of mesh. We have applied this method in 17 consecutive periampullar neoplasm patients, and none of them developed pancreatic leakage.

MATERIALS AND METHODS

Clinical data

Nine male and eight female patients with periampullar malignancy, aged 38-72 years, were included in this study, including 6 patients with carcinoma of the panceatic head, 6 with distal common bile duct cancer, 3 with ampullar carcinoma and 2 with duodenal carcinoma. All the patients received polypropylene mesh-reinforced pancreatoduodenectomy and the Child's method was used to rebuild the alimentary tract. Initial polypropylene MRP was performed on 16 patients, the other patient received end-to-end inviganation anastomosis during the first operation but developed pancreatic leakage after operation. On the 8th postoperative day, the patient developed massive intraabdominal bleeding and received the second laparotomy along with polypropylene MRP.

Technique

The pancreas was transected with a scalpel on the scheduled line. Hemostasis was secured by suture ligatures with 4-0 polyprolene stitches (Ethicon, Somerville, NJ) or electrocautery. A 3.0 cm cut end of the pancreatic remnant was isolated. A 1.0 cm wide polypropylene mesh (Ethicon, Somerville, NJ) strip was tightly wrapped over the pancreatic stump about 1.0 cm from the cut margin with a few stitches. The mesh was fixed if it could not be moved with force. If the main pancreatic duct was identified, a stent tube was inserted into the main pancreatic duct and fixed with suturing thread (Figure 1A). Then, the pancreatic stump and the free margin of jejunum were brought together. A posterior row of continuous running sutures using a 3-0 polyprolene stitch was placed between the inner edge of the mesh and jejunum. The sutures were passed carefully through the

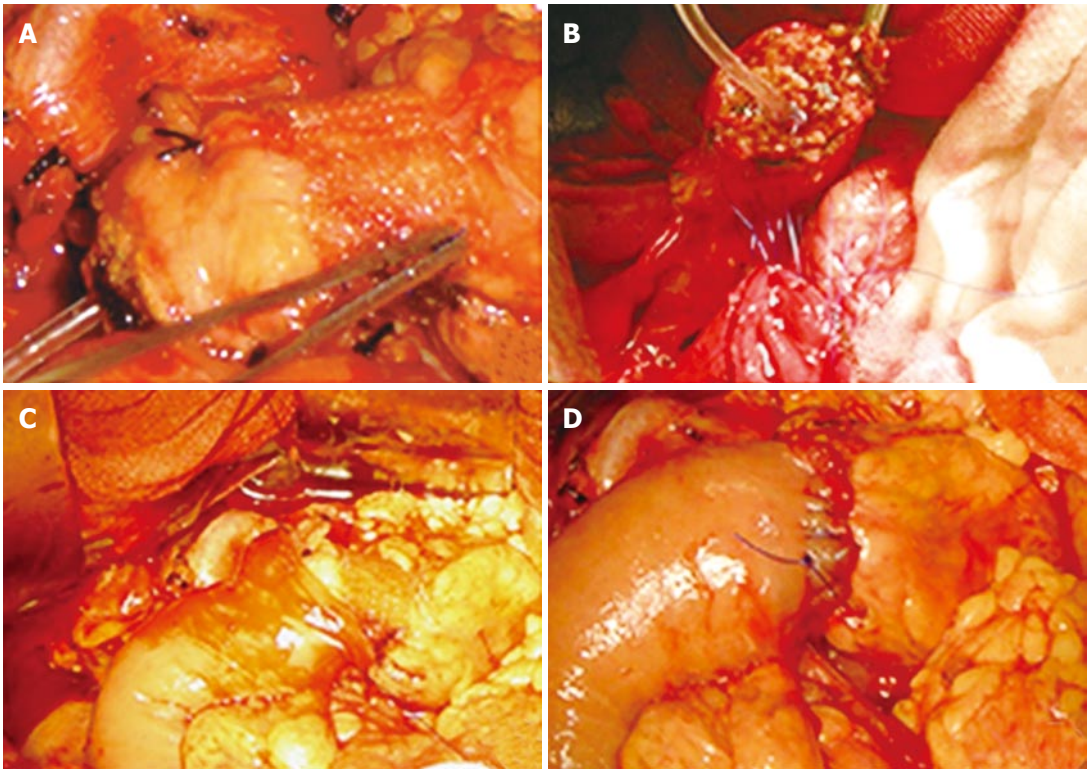

Figure 1 Fixation of mesh around the pancreatic stump (**A**); running suture on the posterior wall of pancreas with a stitch placed between inner edges of the mesh and jejunum (**B**); invagination of the pancreatic stump into the jejunum (**C**); running suture on the anterior wall of the pancreas with a stitch placed between inner edges of the mesh and jejunum (**D**).

mesh, pancreatic capsule and full thickness of jejunum. We began at the farthest point on the cranial side of the pancreatic stump and the caudal side was ligated with six to eight sutures (Figure 1B). After the posterior sutures were completed, they were gently pulled to invaginate the pancreatic stump into the jejunum (Figure 1C). The opposite end of the pancreatic duct stent tube was traversed through sites where bilioenteric anastomosis was performed. Finally, the continuous sutures were extended anteriorly using the same stitch. The sutures were tied after the tightness of the suture line was confirmed. As a result, the jejunal stump invaginated the pancreatic stump of about 2.0 cm (Figure 1D). Care was taken to cover the entire mesh in the jejunal lumen. A urinary catheter was inserted into the jejunum, and saline solution was injected to test for a watertight closure. The seromuscular surface of jejunum 1.0 cm from the margin was anchored to the superior and inferior peritoneal attachments of the pancreatic body to minimize the tension. Biliary anastomosis was constructed in an end-to-side fashion 15 cm distal from the pancreaticojejunostomy. Then a Jackson-Pratt drainage tube was placed near the anastomosis. Daily output and amylase content of abdominal drainage were measured after operation. Pancreatic leakage was defined as the persistent amylase-rich (more than three times the serum concentration) drainage output excess 50 mL/d[1,2]. Prophylactic octreotide was not used.

RESULTS

The mean operative time for polypropylene MRP was 22 min (ranged 10-25 min). No pancreatic leakage was observed under the pressure of 20 cmH_2O during operation. The abdominal drainage output was less than 50 mL in 14 patients, and the amylase content in drainage was within normal range (ranged 46-98 IU/L). The abdominal drainage was removed 4-5 d after the operation, and the patients recovered well and were discharged on the 10th postoperative day. Two patients had a high volume of abdominal drainage during the first 3 postoperative days, but the amylase content in the abdominal drainage was within normal range. The abdominal drainage was removed on the 7th postoperative day and the patients recovered well. A 55-year old male patient received pancreaticojejunectomy for distal common bile duct with an end-to-end invagination anastomosis. The pancreas was soft and fragile. The abdominal drainage output was > 100 mL/d after operation and the amylase content was > 2000 IU/L. Pancreatic anastomotic leakage was identified. The patient had no fever or abdominal pain and received conservative therapy initially. On the 8th postoperative day, fresh blood was drained from the abdominal drainage tube and the patient received the second laparotomy. During the operation, laceration of pancreatic tissue was found on the suture of anastomosis with massive fluid collection. The bleeding came from the splenic artery involved in the fluid collection. After the bleeding was stopped, the pancreas was found to be edematic and fragile. In order to make a direct suture on the pancreas, we decided to perform polypropylene MRP. The patient recovered well after the second operation without pancreatic leakage. All patients were followed up in the outpatient clinic and no adverse events occurred.

DISCUSSION

Since Whipple introduced pancreaticoduodenectomy, it has become a standard procedure for malignant and

benign disorders of the pancreatic head and periampullary region[3-5]. Although the mortality from the surgical procedure has come down considerably during the past three decades[6], it is still higher than that of other radical procedures for abdominal malignancy[7-9]. Pancreatic anastomotic leakage is still the most important determinant of its morbidity, with an incidence of 2%-14%[10,11] and mortality of 28%[12,13]. The etiology of pancreatic anastomotic leakage covers several aspects, including quality of pancreatic tissue, size of major pancreatic duct, exocrinal status of pancreas, general condition and nutritional status of the patient, skill of the surgeon and method of pancreaticojejunostomy[6,14-16]. Among them, method of pancreaticojejunostomy seems to be the key factor[17]. Different methods for pancreaticojejunostomy have been reported in the literature[19], including end-to-side anastomosis, duct-to-mucosa anastomosis, or end-to-end or end-to-side invagination anastomosis. The suturing techniques for anastomosis include running or interrupted suture, single layer or double layers suture[18-20]. But all these methods cannot absolutely prevent the leakage, the incidence of leakage of the most widely applied end-to-side invagination anastomosis is still as high as 11%[21]. No consensus on the choice of anastomotic technique has been reached, and currently each technique finds its application among different groups of surgeons[22].

It is well known that the incidence of pancreatic leakage is higher in patients with a soft and normal pancreatic parenchyma because it is prone to develop parenchymal laceration from shear forces applied during tying of the sutures, especially while performing suturing on the posterior wall of the pancreas. In patients with normal pancreatic parenchyma, the incidence of leakage is 12% to 28%, compared with 5% to 9% in those considered to have pancreatic fibrosis[23]. The efferent loop filled with bile and pancreatic juice also increases the shear force[24]. Some retrospective or prospective studies also suggested that technical modifications may reduce the leakage rate[25-27], suggesting that if the pancreas is soft with a narrow duct, it would be most secure when the pancreaticojejunal anatomosis is intraluminal into the jejunum by invaginating the pancreatic stump.

Polyprolene mesh-reinforced pancreaticojejunostomy was developed based on the binding pancreaticojejunostomy described by Peng *et al*[28,29], who reported a widely invaginated end-to-end anastomosis with ablated jejunal mucosa. Our procedure with single layer continuous sutures is less complicated than the binding pancreaticojejunostomy. The success of this technique may be due to the following four aspects. First, the mesh forms a safe "clothing" around the remnant pancreas for anchoring sutures, thus preventing the possibility of parenchymal laceration and bleeding from the sutures in soft pancreatic parenchyma caused by the suture. We identified the advantages of a new technique in the case where a secondary polypropylene MRP was received for leakage from the first operation site. During the second operation, the pancreatic parenchyma was severely edematic and fragile, making the direct suture impossible. However, it was easy and convenient to perform polypropylene MRP on this patient, and no leakage occurred postoperatively. Second, sine the shape of the pancreatic stump can be modified and reduced by the mesh, it is more convenient to make an invagination. Third, the posterior single layer continuous sutures are simple and require less time. Fourth, it is very convenient to perform polypropylene MRP under different conditions and the time required is less than single end-to-end invagination. The mesh in the anastomosis can promote fibroblast attachment and enhance the anastomotic healing process[30,31]. However, it still needs further confirmation.

An ideal pancreaticojejunal anastomosis should be safe and convenient. Moreover, laparoscopy is more and more widely applied by general surgeons, and the convenience of a surgical procedure should be considered. The use of polypropylene MRP ensures a tight seal for any type of pancreatic stump regardless of the pancreas consistency, thus a more secure and reliable anastomosis can be obtained. The preliminary results are very encouraging. However, an appropriate prospective study in randomized patients is needed. Up to date, we have not observed any adverse effect of polypropylene MRP, but a long following-up time is needed to confirm it.

COMMENTS

Background

Pancreaticoduodenectomy is the standard procedure for malignant and benign disorders of the pancreatic head and periampullary region. Mortality and morbidity of this procedure are still higher than other radical procedures for abdominal malignancy. Pancreatic anastomotic leakage is still the most important determinant of its morbidity.

Research frontiers

The method of pancreaticojejunostomy is the key factor for pancreatic anastomotic leakage. We designed a new technique of polypropylene mesh-reinforced pancreaticojejunostomy to prevent pancreatic leakage.

Innovations and breakthroughs

Up to date, all the existing methods of pancreaticojejunostomy cannot perish anastomotic leakage. Our new method is effective in preventing pancreatic leakage.

Applications

This technique is safe and can be applied in pancreaticoduodenectomy under all conditions even though the pancreas is very fragile. If its advantage and disadvantage can be proved by large clinical trials, it can be used as one of the standard procedures.

Terminology

Pancreaticoduodenectomy: excision of the pancreatic head and the encircling loop of the duodenum to which it is connected. Pancreaticojejunostomy: surgical anastomosis of the pancreatic duct or the divided end of transected pancreas with the jejunum.

Peer review

This article seems to be a challenging artifice to reduce postoperative complication in pancreatic surgery. The results are encouraging and the procedure can be used as a standard method of pancreaticojejunostomy.

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RAPID COMMUNICATION

Correlation of matrix metalloproteinase suppressor genes RECK, VEGF, and CD105 with angiogenesis and biological behavior in esophageal squamous cell carcinoma

Sheng-Lei Li, Dong-Ling Gao, Zhi-Hua Zhao, Zong-Wen Liu, Qiu-Min Zhao, Jin-Xia Yu, Kui-Sheng Chen, Yun-Han Zhang

Sheng-Lei Li, Zhi-Hua Zhao, Dong-Ling Gao, Zong-Wen Liu, Qiu-Min Zhao, Jin-Xia Yu, Kui-Sheng Chen, Yun-Han Zhang, Department of Pathology, The First Affiliated Hospital Zhengzhou University; He'nan Key Laboratory of Tumor Pathology, Zhengzhou 450052, He'nan Province, China
Supported by The "Tenth Five-Year Plan" Research Foundation for the Key Constructional Project ("211 Project") of Zhengzhou University, He'nan Province, China
Correspondence to: Yun-Han Zhang, Department of Pathology, The First Affiliated Hospital, Zhengzhou University; Henan Key Laboratory of Tumor Pathology, Zhengzhou 450052, He'nan Province, China. yhzhang@zzu.edu.cn
Telephone: +86-371-65168738 Fax: +86-371-66658175
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Abstract

AIM: To explore the expression of reversion inducing cysteine-rich protein with Kazal motifs (RECK), vascular endothelial growth factor (VEGF) and endoglin (CD105) protein and its correlation with occurrence, development, invasion and metastasis in esophageal squamous cell carcinoma (ESCC).

METHODS: Streptavidin-peroxidase (SP) immunohistochemistry was used to detect expression of RECK and VEGF in 62 cases of ESCC, 31 cases of adjacent atypical hyperplastic epithelium and 62 cases of normal esophageal epithelium. CD105 Mb was used to assess microvessel density (MVD).

RESULTS: The expression of RECK was closely correlated with histological grade, infiltrative depth and lymphatic metastasis in ESCC ($P < 0.05$). The expression of RECK decreased during cancer development: normal esophageal epithelium (85.5%, 53/62), adjacent atypical hyperplastic epithelium (71.0%, 22/31), and carcinoma (59.7%, 37/62). There was a significant difference among the groups ($P < 0.05$). The expression of VEGF protein was closely correlated with infiltrative depth and lymphatic metastasis in ESCC ($P < 0.05$). The expression of VEGF protein increased during cancer development: normal esophageal epithelium (29.0%, 18/62), adjacent atypical hyperplastic epithelium (54.8%, 17/31), and carcinoma (67.7%, 42/62). There was a significant difference among the groups ($P < 0.05$). MVDCD105 increased in accordance with histological grade, but there was no significant difference (grade Ⅰ, 36.92 ± 10.85; grade Ⅱ, 37.65 ± 9.50; and grade Ⅲ, 38.06 ± 12.19). The MVDCD105 was closely correlated with infiltration and lymphatic metastasis in ESCC ($P < 0.05$). The expression of RECK was inversely correlated with the expression of VEGF and CD105.

CONCLUSION: RECK, VEGF and CD105 play important roles in the infiltration, metastasis and carcinogenesis in esophageal carcinoma. Angiogenesis in ESCC may be promoted by over-expression of CD105.

Key words: Reversion inducing cysteine rich protein with Kazal motifs; Vascular endothelial growth factor; CD105; Esophageal squamous cell carcinoma; Immunohistochemistry; Microvessel density

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INTRODUCTION

Reversion inducing cysteine-rich protein with Kazal motifs (RECK) is a recently discovered tumor suppressor gene with a special function of inhibiting matrix metalloproteinase (MMP) expression and activity, which serves as an MMP inhibitor[1]. Expression of the RECK gene is closely related to tumor invasion and metastasis and angiogenesis. Previous studies indicate that the level of RECK gene expression is inversely correlated to tumor invasiveness in liver cancer, pancreatic cancer, mammary cancer and pulmonary carcinoma, and for patients with higher RECK gene expression, the prognosis is sometimes apparently better than that of patients with low expression[2-5]. No studies have been published in China or abroad on the correlation of the RECK gene with invasion and metastasis of esophageal cancer, and the relationship between RECK, vascular endothelial growth factor (VEGF)

and endoglin (CD105) expression. The streptavidin–peroxidase (SP) immunohistochemistry method was used to perform a combined test on expression of RECK, VEGF and CD105 gene in tissues from 62 cases of esophageal squamous cell carcinoma (ESCC), 31 cases of para-carcinoma atypical hyperplasia, and 62 specimens of normal esophageal mucous membrane, to establish the role of RECK, VEGF and CD105 in the generation and development of esophageal cancer, so as to ascertain the molecular index for early diagnosis and prognosis judgment.

MATERIALS AND METHODS

Materials

Resection specimens from 62 cases of esophageal cancer were collected from the Municipal Cancer Hospital of Anyang, Henan Province, China from 26 February to 16 March, 2006, which is one of the most epidemic regions for esophageal cancer. No patients had a history of chemotherapy, radiotherapy or immunotherapy. The specimens were taken from 36 male and 26 female patients aged 38-75 years (average 60.6 ± 9.5), who were all verified to have ESCC by histopathological examination. The histological grading included Class Ⅰ (15 cases), Class Ⅱ (25 cases) and Class Ⅲ (22 cases); 20 cases were accompanied with lymphatic metastasis, and 42 cases had no lymphatic metastasis. The depth of invasion was divided into two groups that consisted of seven cases with invasion of the superficial muscularis, and 55 with invasion of the deep muscularis or fibrous membrane. All the samples were taken from within 3 cm of the tumor focus, as well as from three areas of distal normal mucous membrane, and were fixed with 40 g/L paraformaldehyde solution, normally dehydrated, embedded in paraffin, and serial sections were cut to a thickness of 4-6 μm, and used for hematoxylin and eosin and immunohistochemistry staining. Mouse anti-human RECK monoclonal antibody (mAb) and anti-human VEGF mAb were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and mouse anti-human CD105 single clone antibody and the SP immunohistochemistry kit were purchased from Beijing Zhongshan Golden Bridge Biotech Development (China).

Methods

The SP immunohistochemistry SP method was employed. RECK, VEGF and CD105 mAbs were diluted 1:100, stained with diaminobenzidine, and counterstained with hematoxylin, strictly in accordance with the instructions, and PBS solution was used as a negative control replacing primary antibody. RECK- and VEGF-positive signals all show brown granule-like materials that are located in the cytoplasm. Under a high-power magnifying glass, five fields of vision (FOVs) were randomly selected (for each FOV, there were no fewer than 200 cells), and the results were interpreted in accordance with the percentage of cells and depth of stain[6]. (1) Scored in accordance with depth of staining of cells in a section: 0, no cell coloration; 1, light yellow; 2, brown; 3, tan. (2) Scored in accordance with the percentage of positive cells in like-kind cells: 1, < 30%; 2, 30%-70%; 3 > 70%. The product of (1) and (2) was used as the total score, where 0-1 indicates a negative score (-), 2-3 a weak positive score (+), and ⩾ 4 a positive score (++). Tumor MVD measurement was done using the methods reported by Weidner[7], i.e. any brown endothelial cell or cell cluster is used as a vessel. We first observed all the FOVs for a given section under a low-power magnifying glass to find the highest density area of tumor vessels, and then we counted the number of microvessels in three FOVs under a high-power magnifying glass; the average values were then used as the MVD.

Statistical analysis

SPSS 10.0 statistical software was used and the χ^2 test, single factor analysis of variance, *t* test were applied, correlation test was applied, Spearman correlation analysis. The test level was $\alpha = 0.05$.

RESULTS

RECK expression in ESCC tissues and correlation with clinical and biological behavior

RECK expression was mainly located in the cytoplasm of tumor cells, and appeared as light to dark yellow (Figure 1A). RECK expression increased sequentially as ESCC developed: normal tissue (59.7%, 37/62), para-carcinoma atypical hyperplastic tissue (71.0%, 22/31), and ESCC (85.5%, 53/62), and comparison between the groups indicated a significant difference ($\chi^2 = 10.331$, $P < 0.01$) (Table 1). RECK expression was related to histological grading, invasion depth and lymphatic metastasis (the respective values of χ^2 were 10.422, 8.550 and 4.751; average $P < 0.05$) (Table 2).

VEGF expression in ESCC tissues and correlation with clinical and biological behavior

VEGF staining was located in the cytoplasm, and appeared as light to dark yellow (Figure 1B). VEGF expression decreased sequentially as ESCC developed: normal mucous membrane (67.7%, 42/62), para-carcinoma atypical hyperplastic tissue (54.8, 17/31), and carcinoma (29.0% 18/62); and comparison between the groups indicated a significant difference ($\chi^2 = 18.994$, $P < 0.05$) (Table 1). VEGF expression was not related to histological grading ($P > 0.05$), but was related to depth of invasion and lymphatic metastasis (the respective values of χ^2 were 10.319 and 6.693; average $P < 0.05$) (Table 2).

Correlation of MVDCD105 with differentiation and metastasis in ESCC

CD105 expression was mainly located in the cytoplasm of vascular endothelial cells of tumor stroma, and appeared as light to dark yellow granules (Figure 1C). In grade Ⅰ, Ⅱ and Ⅲ ESCC tissues, MVDCD105 tended to increase as the degree of cancer tissue differentiation decreased: grade Ⅰ, 37.87 ± 3.60; grade Ⅱ, 37.44 ± 3.99; and grade Ⅲ, 39.00 ± 4.47), but there was no significant difference between the results ($F = 0.885$, $P > 0.05$) (Table 1). In cancer tissues with lymphatic metastasis, MVD (41.00 ±

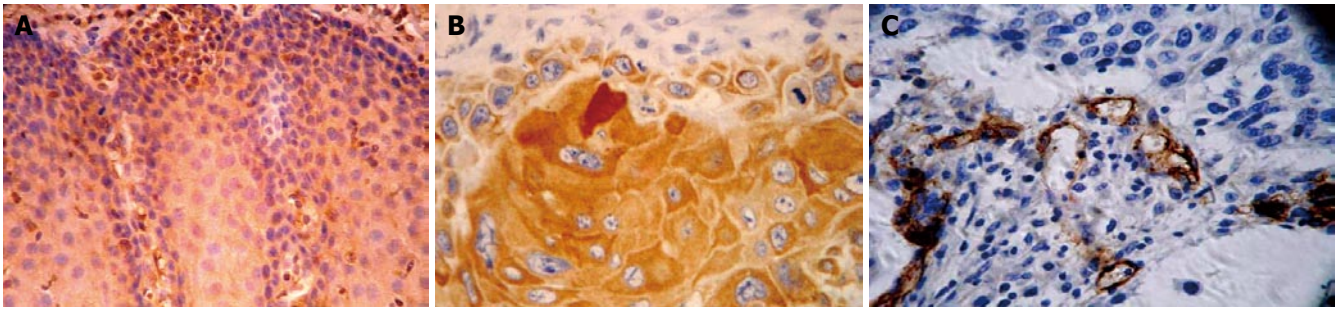

Figure 1 **A**: Expression of RECK in the normal esophageal epithelium; **B**: Expression of VEGF in ESCC; **C**: Expression of CD105 in ESCC (SP, × 400).

Table 1 RECK and VEGF expression in ESCC, atypical hyperplasia and normal mucous membrane tissues

		RECK			χ^2	*P*	VEGF			χ^2	*P*
	n	-	+	Positive ratio (%)			-	+	Positive ratio (%)		
Normal mucous membrane tissues	62	9	53	85.5			44	18	29		
Atypical hyperplasia	31	9	22	71.0	10.331	0.006	14	17	54.8	18.994	0.000
ESCC	62	25	37	59.7			20	42	67.7		

Table 2 Correlation of RECK and VEGF expression with clinical and biological behavior of ESCC

	n	RECK			χ^2	*P*	VEGF			χ^2	*P*
		-	+	Positive ratio (%)			-	+	Positive ratio (%)		
Histology grading											
I	15	1	14	93.3			8	7	46.7		
II	25	11	14	56	10.422	0.005	7	18	72.0	4.014	0.134
III	22	13	9	40.9			5	17	77.3		
Invasion depth											
Superficial muscularis	7	0	7	100			6	1	14.3		
Deep muscularis	55	25	30	54.5	8.550	0.021	14	41	74.5	10.319	0.001
Lymph metastasis											
N	42	13	29	69	4.751	0.029	18	24	57.1	6.693	0.010
Y	20	12	8	40			2	18	90.0		

3.26) was higher than that without metastasis (36.33 ± 3.76). MVD (38.80 ± 3.60) of cancer tissues with invasion of the deep muscularis was higher than that of the superficial muscularis (32.57 ± 3.46), and the difference was significant (Table 3).

Correlation of RECK, VEGF and CD105 expression in ESCC

RECK expression was inversely correlated to that of VEGF. In tissues with positive RECK expression, VEGF expression rate was 51.4% (19/37), and in tissues negative for RECK expression, VEGF expression rate was 92.0% (23/25). Comparison between the groups indicated a significant difference (γ_s = -0.427, $P < 0.01$). In tissues with positive RECK expression, MVDCD105 was 35.76 ± 9.42, and tissues negative for RECK expression, MVDCD105 was 41.59 ± 10.80. Comparison between the groups indicated a significant difference (t = -2.969, $P < 0.01$) (Table 4).

DISCUSSION

The RECK gene were discovered by Takahashi *et al*[1] in NIH3T3 cell lines transfected by the v-Ki-Ras gene, which is located on chromosome 9p13-p12 and encodes a membrane-anchored glucose protein with a relative molecular mass of 110 000. RECK gene has high expression in normal tissues, but no expression in various tumor cell lines and cells affected by cancer genes such as Ras. Many cancer genes such as ras, fos and myc can all decrease the expression of the RECK gene[8,9], which indicates that the RECK gene may be a negatively adjusted target jointly acted upon by cancer genes, and the proper expression of the RECK gene can inhibit angiogenesis[10-12]. It has been shown that the RECK gene is closely related to the prognosis of liver cancer, pancreatic cancer and mammary cancer, and prognosis of patients positive for RECK expression is better than that of those with negative expression[2-5]. The effects of RECK during the course of tumor generation and metastasis may be more widespread than has been discovered to date. It is now considered that RECK may affect tumor invasion and metastasis through inhibiting tumor angiogenesis, and is thus a cancer-inhibiting gene[12-14]. The results of this experiment indicate that the RECK gene is expressed in normal esophageal tissues, esophageal para-carcinoma atypical hyperplastic

Table 3 Correlation of CD105 expression with the clinical and biological behavior of ESCC (reciprocal) (mean ± SD)

	CD105			
	n	MVD	*t*/*F*	*P*
Histology grading				
Ⅰ	15	37.87 ± 3.60		
Ⅱ	25	37.44 ± 3.99	F = 0.885	0.418
Ⅲ	22	39.00 ± 4.47		
Invasion depth				
Superficial muscularis	7	32.57 ± 3.46	-4.326	0.000
Deep muscularis	55	38.80 ± 3.60		
Lymph metastasis				
N	42	36.33 ± 3.76	4.760	0.000
Y	20	41.00 ± 3.26		

Table 4 Correlation of RECK, VEGF and CD105 expressions in ESCC (mean ± SD)

RECK	*n*	VEGF		γ_s	*P*	MVD	*t*	*P*
		+	-					
+	37	19	18	-0.427	0.001	36.00 ± 3.80	-2.969	0.004
-	25	23	2			39.10 ± 3.86		

tissues and cancer tissues, but its expression level in cancer tissue is significantly lower. This indicates that tumors with low RECK expression have greater invasive capacity.

VEGF is a polypeptide cell factor discovered in recent years (also called vascular permeability factor), which has a double function: (1) it directly stimulates vascular endothelial cell reproduction through its receptor, and induces production of the proteolytic enzyme interstitial collagenase and tissue factor to promote angiogenesis; and (2) it increases vascular permeability, promotes exosmosis of fibrinogen to cause tumor interstitial edema and extracellular matrix changes, and consequently provides a basis for tumor invasion and metastasis[15,16]. VEGF is an angiogenic factor that has been studied at the most with the most specific effect at present, and which is related to generation and metastasis of various human tumors[17-23]. The results of this study show that VEGF expression is inversely related to ESCC lymphatic metastasis, i.e. the positive protein rate of VEGF for the lymph metastasis group significantly higher than that of no lymph metastasis group, which indicates that the positive protein expression of VEGF maybe correlated with metastasis of ESCC lymph. The results of this study also show that VEGF expression is not obviously related to the degree of tumor differentiation, which indicates that VEGF expression is not related to ESCC histology.

CD105 is a gene located in human chromosome 9q34, which is a homotype dipolymer membrane glycoprotein with a molecular mass of 180 kDa, participating in signal transmission inverting growth factor β (TGF-β) receptor and angiogenesis. Previous studies indicate that strong expression of CD105 in tumor-related, newly generated vascular endothelium is a more accurate index for judging endothelial reproductive state, and it is closely related to tumor generation and metastasis[24-27]. Contrasting with endothelial cell markers such as CD31, CD34 and Ⅷ factor-related antigens, the difference is that CD105 as a marker of angiogenesis is only strongly expressed in vascular endothelial cells of tumor tissues at the reproduction stage (i.e. newly generated vascular endothelium), but it is not expressed in the blood vessels of normal tissues[28-30]. A quantitative test has been carried out on the MVD of newly generated vessels in ESCC with anti-CD105 single clone antibody, the results of which indicated that MVD is positively correlated with depth of invasion and lymphatic metastasis in ESCC. That is, the MVD of tumors with deep invasion is significantly higher than that with superficial invasion, and the MVD of tumors with lymphatic metastasis is significantly higher than that of those without metastasis; however, MVD is not related to histological grading. Thus, the fixed vessel quantity in tumors is considered as a significant and separate prognosis index. The level of angiogenesis in cancer can be evaluated through MVD measurement[31], and MVD measurement in ESCC may help to judge its potential for invasion and metastasis. The MVD in tumor tissues was tested with CD105, the results of which indicate that RECK expression is inversely related to tumor angiogenesis; further, MVDCD105 for tumors with high RECK expression is obviously lower than that for those with low RECK expression, which indicates that RECK can inhibit angiogenesis. There is a co-adjustable mechanism between MVDCD105 and RECK.

Angiogenesis inhibition by RECK has also been verified in some clinical studies that have discovered that MVD in tumor tissues is inversely related to RECK expression[10,32]. However, such an inverse correlation only occurs when the expression of VEGF is much higher, i.e. for tumors with higher expression of VEGF, the influence of RECK also increases, which indicates that RECK can inhibit VEGF-induced angiogenesis. The results indicate that RECK expression is closely related to tumor prognosis. Also, the inhibitory effects of RECK on tumor angiogenesis have a certain pertinence. The results of this study indicate that positive expression of RECK is inversely correlated with VEGF expression and MVD. In cases with negative RECK expression, VEGF expression and MVD are significantly higher than in cases with positive RECK expression ($P < 0.05$). This indicates that decreasing or losing RECK expression may increase VEGF expression, which consequently promotes tumor angiogenesis, and provides the conditions for the generation and metastasis of ESCC. This study also discovered that VEGF expression is consistent with MVD, i.e. if VEGF expression is high, MVD will rise accordingly ($P < 0.05$), and the VEGF's positive stained cells at the front of tumor infiltration, which are consistent with CD105's expression positions, which further verifies that VEGF is an angiogenesis factor with specific effects, and can specifically promote tumor angiogenesis. This study shows that decreasing or losing RECK expression and increasing VEGF expression are two of the significant events during the generation and development of ESCC, and RECK, as a cancer-inhibiting gene, may inhibit angiogenesis in ESCC, through affecting the signal transmission path of VEGF expression. The combined test on RECK, VEGF and MVD can be used as an

objective index to determine the invasion and metastasis capabilities of ESCC, which is of great significance for judging prognosis.

COMMENTS

Background

The RECK gene was discovered by Takahashi *et al* in NIH3T3 cell lines transfected by the v-Ki-Ras gene, which plays an important role in regulating MMPs and participating in tumor invasion, metastasis and angiogenesis.

Research frontiers

No studies have been published in China or abroad on the correlation of the RECK gene with invasion and metastasis of esophageal cancer, and the relationship between RECK and expression of VEGF and CD105.

Related publications

Expression of RECK gene is closely related to tumor invasion and metastasis and angiogenesis. Previous studies indicate that RECK gene expression is inversely correlated with tumor invasiveness in liver cancer, pancreatic cancer, mammary cancer and pulmonary carcinoma, and for patients with higher RECK gene expression, the prognosis is apparently better than that of patients with low RECK expression. Therefore, RECK is considered to be an cancer-inhibitory gene, which can affect tumor metastasis through inhibiting the activity of MMPs and angiogenesis.

Innovations and breakthroughs

No reports on this subject have been published in China or abroad. The immunohistochemistry SP method was used to perform a combined test on expression of RECK, VEGF and CD105 genes in 62 cases of ESCC, 31 specimens of para-carcinoma atypical hyperplastic tissues, and 62 specimens of normal esophageal mucous membrane, to ascertain the role of RECK, VEGF and CD105 in the generation and development of esophageal cancer, so as to establish a molecular index for early diagnosis and prognosis judgment.

Applications

The further investigation of RECK helps us understand more about the biological behavior of esophageal carcinoma and it gives us a new guide for earlier diagnosis and therapy of esophageal carcinoma. RECK may be a molecular target for the early diagnosis and prognostic judgment of esophageal carcinoma.

Terminology

RECK is a new cancer-inhibiting gene first discovered in NIH3T3 cell lines transfected by v-Ki-Ras.

Peer review

This manuscript reports expression of three genes in ESCC, in which they appear to have prognostic value as they are correlated with histological grade, lymphatic metastasis and invasion.

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RAPID COMMUNICATION

DNA methyltransferase 3B promoter polymorphism and its susceptibility to primary hepatocellular carcinoma in the Chinese Han nationality population: A case-control study

Ying Wu, Ju-Sheng Lin

Ying Wu, Ju-Sheng Lin, Institute of Liver Diseases, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Correspondence to: Ju-Sheng Lin, Professor, Institute of Liver Diseases, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China. jslin@tjh.tjmu.edu.cn
Telephone: +86-27-83663661 Fax: +86-27-83663661
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Abstract

AIM: To investigate the correlation between C/T single nucleotide polymorphism (SNP) in the promoter of the DNA methyltransferase 3B (*DNMT3B*) gene and risk for development and progression of primary hepatocellular carcinoma (HCC).

METHODS: One hundred case subjects were selected consecutively from Tongji Hospital (Wuhan, China). from March to November 2006. They did not receive radiotherapy or chemotherapy for newly diagnosed and histopathologically confirmed HCC. One hundred and forty control subjects having no history of cancerous or genetic diseases were healthy volunteers to Wuhan Blood Center in the same period. Frequency was matched for sex, age, alcohol consumption and cigarette smoking status of the case subjects. C/T polymorphism of the *DNMT3B* promoter was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing analysis. The association between genotypes of *DNMT3B* and clinicopathological parameters among cases was also studied.

RESULTS: The CC genotype was not detected in both HCC patients and controls. In control subjects, the frequency of TT and CT genotypes was 99.3% and 0.7% respectively, and that of T and C alleles was 99.6% and 0.4% respectively. The frequency of CT genotype was higher in HCC (3.0%). The frequency of T and C alleles was 98.5% and 1.5% respectively. However, the genotype and allelotype distribution in HCC patients was not significantly different from that in controls.

CONCLUSION: C/T polymorphism is not associated with the increased risk of HCC. *DNMT3B* genetic polymorphism is variable in different races, ethnic groups or geographic areas. Further study is needed to clarify the role of *DNMT3B* SNP in the development of HCC among other populations.

Key words: DNA methyltransferase; Single nucleotide polymorphism; Susceptibility; Primary hepatocellular carcinoma

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) occurs frequently in Southeast Asia, especially in China. It ranks second and approximately accounts for 42.5% of all malignancies worldwide[1].

Although hepatitis B virus (HBV) is the major cause of HCC, only a fraction of patients with chronic HBV infection develop HCC during their lifetime, suggesting that genetic and epigenetic factors are important in determining individuals' susceptibility to HCC.

DNA methylation plays an important role in chromatin structure stability, genome integrity, modulation of tissue-specific gene expression, embryonic development, genomic imprinting, X-chromosome inactivation and is essential for the development of mammals[2,3]. DNA methylation is mediated by DNA methyltransferases (DNMTs), of which three active forms have been identified: DNMT1, DNMT3A and DNMT3B. DNMT1 is thought to be primarily responsible for maintaining pre-existing methylation patterns after DNA replication because of its preference to hemimethylated DNA substrates and targeting replication foci. DNMT3A and DNMT3B have an equal preference to hemimethylated and unmethylated DNA substrates, and therefore they are believed to be principally required for *de novo* methylation[4-6]. Recent studies have shown that three different mechanisms play a role in the effect of methylation: global hypomethylation, hypermethylation of individual gene segments and deregulated expression of DNA methyltransferases. Changes in the methylation pattern correlate with the

development of cancer. *De novo* hypermethylation in promoter CpG islands has been identified as a possible mechanism for tumor suppressor gene and DNA repair gene inactivation of in human cancer cells[7-12]. DNMT3B, regarded as a *de novo* DNA methyltransferase, is thought to play an important role in the generation of aberrant methylation in carcinogenesis[13,14].

DNMT genes are up-regulated in various human cancers, including HCC[15-20]. Significant over-expression of DNMT3B is observed in tumor tissues while over-expression of DNMT1 and DNMT3A is more modest[15,18,20].

A C-to-T transition polymorphism (C46359T, GenBank accession no. AL035071) in the promoter region of the *DNMT3B* gene, -149 base pairs from the transcription start site, is reported to greatly increase promoter activity. Many reports have shown that the C/T polymorphism is associated with an increased risk for lung cancer and decreased postsurgical survival in patients with small cell carcinoma of the head and neck. Carriers of T allele, particularly heterozygotes, have a significantly increased risk for such cancers[21-24].

Several polymorphic genes are reported to be correlated with modification of susceptibility to HCC. To our knowledge, the association between *DNMT3B* polymorphism and development of HCC has not been reported. Since the *DNMT3B* promoter polymorphism that is responsible for regulating genomic methylation is possibly associated with an increased risk for cancers, we evaluated the relationship between *DNMT3B* C46359T polymorphism and risk of HCC in a hospitalization-based case-control study in a Chinese Han nationality population.

MATERIALS AND METHODS

Subjects

One hundred case subjects were selected consecutively from Tongji Hospital (Wuhan, China) from March to November 2006. They did not receive radiotherapy or chemotherapy for newly diagnosed and histopathologically confirmed HCC. One hundred and forty control subjects having no history of cancerous or genetic diseases were healthy volunteers to Wuhan Blood Center in the same period. Frequency was matched for sex, age, alcohol consumption and cigarette smoking status of the case subjects. All the cancer patients and control subjects were unrelated Han nationality individuals from Wuhan or from its surrounding regions. Blood was taken from all recruits who consented to the epidemiology survey. Each subject was scheduled for an interview after informed consent was obtained, and a structured questionnaire was used to collect information on demographic data and risk factors, such as hepatitis B infection history and family history of any cancers.

DNMT3B genotyping

Genomic DNA was extracted from peripheral blood lymphocytes by proteinase K digestion and phenol-chloroform extraction. DNMT3B C/T polymorphism was determined by PCR-RFLP. The primers of 5'-TGCTGT GACAGGCAGAGCAG-3' (nt 46151-46170) and 5'-GG TAGCCGGGAACTCCACGG-3' (nt 46530-46511) were synthesized as previously described[21]. This 380-bp target DNA fragment contains the upstream region and the first exon of the *DNMT3B* gene. Amplification reaction was carried out in a 25 μL PCR mixture containing 50-200 ng of genomic DNA, 12.5 pmol of each primer (Shanghai Sangon Company), 0.1 mmol/L each deoxynucleotide triphosphate, 1 × PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, and 0.1% Triton X-100), 2.0 mmol/L $MgCl_2$, and 1.25 U Taq polymerase (Beijing Sbsbio Company). The PCR profile consisted of an initial denaturation at 95℃ for 5 min, 35 cycles of denaturation at 95℃ for 30 s, annealing at 65℃ for 30 s and extension at 72℃ for 30 s and a final extension at 72℃ for 10 min. The 380-bp product was then digested with *Bln* I (TaKaRa Biotechnology Company) for 12-16 h in 37℃ bath water. The digested product was separated by vertical electrophoresis at 200V of constant voltage for 1.5 h through 8% polyacrylamide gel (29:1) and silver staining, and then determined under ultraviolet irradiation. The variant T allele has a *Bln* I restriction site that results in two bands (207 bp and 173 bp), and the wild-type C allele lacks the *Bln* I restriction site, thus producing a single 380-bp band (Figure 1). More than 10% of the samples were randomly selected for repeated assays, and the results were 100% concordant. Restriction fragment length polymorphism (RFLP) analysis was confirmed by PCR-based sequencing with an Applied Biosystem automated sequencer (Figure 2).

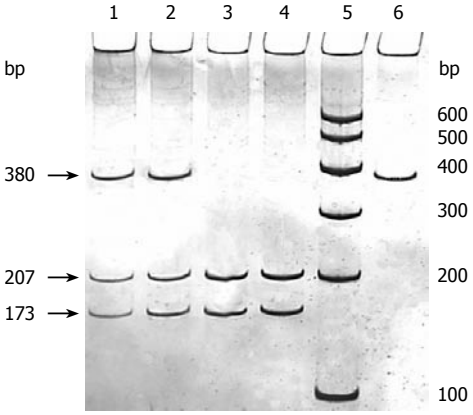

Figure 1 PCR-RFLP-polyacrylamide gel electrophoresis for genotyping of DNMT3B promoter C46359T. Lanes 1 and 2: CT heterozygote; lanes 3 and 4: TT genotype; lane 5: 100 bp molecular marker; lane 6: PCR product.

Statistical analysis

Data were presented as mean ± SD. A database was developed by Epi Info 6.0 and the analysis of data was accomplished using SPSS 10.0 software. The difference in frequency distributions of genotypes and allelotypes between the two groups was detected by chi-square test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

The characteristics of 100 HCC case patients and 140 control subjects are summarized in Table 1. In the case group, the age ranged 18-70 years, the mean age was (55.86 ± 10.12) years, and the gender ratio was 4:1 while the mean

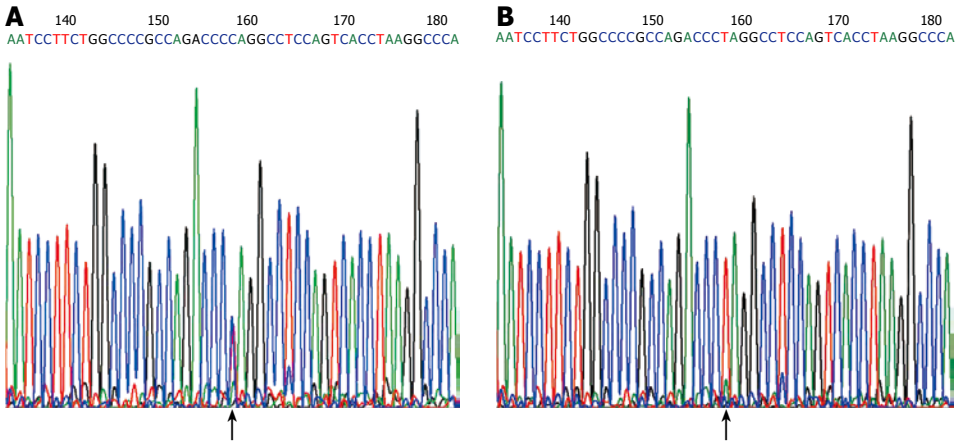

Figure 2 CT genotype (**A**) and TT genotype (**B**) Sequencing-confirmed.

Table 1 Frequency distributions of selected variables in HCC patients and control subjects, *n* (%)

Variable	Case (*n* = 100)	Control (*n* = 140)
Sex		
Male	80 (80)	114 (81.4)
Female	20 (20)	26 (18.6)
Age (yr)	55.86 ± 10.12	57.12 ± 10.01
Smoking status		
Current smoker	60 (60.0)	92 (65.7)
Former smoker	21 (21.0)	26 (18.6)
Non smoker	19 (19.0)	22 (15.7)
Pack/year	11.26 ± 6.25	14.89 ± 7.56
Alcohol consumption		
Current drinker	55 (55.0)	70 (50.0)
Former drinker	19 (19.0)	29 (20.7)
Non drinker	26 (26.0)	41 (29.3)
Alcohol/year	10.01 ± 5.21	13.58 ± 5.09

Table 2 *DNMT3B* genotype and allele frequencies in case patients and control subjects and their association with HCC, *n* (%)

Genotypes	Case patients (*n* = 100)	Control subjects (*n* = 140)
CT	3 (3)	1 (0.7)
TT	97 (97)	139 (99.3)
C allele	3 (1.5)	1 (0.4)
T allele	197 (98.5)	279 (99.6)

$\chi^2 = 1.86$, $P = 0.17$ (genotype); $\chi^2 = 1.84$, $P = 0.17$ (allele).

time of alcohol consumption and cigarette smoking was (10.01 ± 5.21) years and (11.26 ± 6.25) years, respectively. In the control group, the age ranged was 15-68 years, the mean age was (57.12 ± 10.01) years, and the gender ratio was 4.4:1 while the mean time of alcohol consumption and cigarette smoking was (13.58 ± 5.09) years and (14.89 ± 7.56) years, respectively. The two groups had a similar frequency of distribution in age, sex, alcohol consumption and cigarette smoking status. In addition, 95% of the case patients had a chronic infection with HBV but others had no infection with any hepatitis virus.

The *DNMT3B* T allele frequency and genotype distributions in the case patients and control subjects are summarized in Table 2. Only one genotype of CT was found in 140 control subjects (0.7%) and three in 100 case subjects (3.0%), respectively. The variant C allele frequency was 1.5% for the case patients and 0.4% for the control subjects. The CC genotype was not detected in both HCC patients and controls. Although the frequency of CT genotype and C allele was higher in HCC patients than in control subjects, but there was no significant difference ($P > 0.05$).

DISCUSSION

Alterations in DNA methylation can cause changes in gene transcription patterns and also promote mutational events leading to malignant tumors. Recent studies have shown that several mechanisms, including DNA hypomethylation on pericentromeric satellite regions, DNA hypermethylation on CpG islands of genes such as *p16*, *E-cadherin*, and *HIC-1* (hypermethylated-in-cancer), over-expression of DNA methyltransferases, and reduced expression of methyl-CpG-binding proteins play a role in hepatocarcinogenesis[25-27]. It was reported that genetic disruption of both *DNMT1* and *DNMT3B* in human cancer cells results in global and gene-specific demethylation, and abrogation of silencing of tumor suppressor genes. Both the wild type and catalytically inactive *DNMT3B* mutant can suppress rDNA promoter irrespective of its methylation status[28,29], suggesting that altered activities of DNMTs contribute to the generation of aberrant methylation in cancer.

Single nucleotide polymorphisms (SNPs) are the most common form of human genetic variation. SNPs in the promoter region of genes may affect either the expression or the activity of enzymes and therefore may be mechanistically associated with cancer risk. The *DNMT3B* gene contains a C-to-T transition polymorphism (C46359T) in a novel promoter region, -149 bp from the transcription start site, which in *in vitro* assays confers a 30% increase in promoter activity[23]. It was postulated that the T variant might up-regulate *DNMT3B* expression, resulting in a predisposition towards aberrant *de novo* methylation of CpG islands in tumor suppressor and DNA repair genes. These findings encouraged us to examine the relationship between a novel polymorphism in the human *DNMT3B* promoter and risk of HCC.

To select China as a research field to analyze the relationship between *DNMT3B* genetic polymorphisms and HCC is a new attempt. In this study, the *DNMT3B* genetic polymorphism was not susceptible to HCC. The

CC genotype was not detectable in both HCC patients and controls, while the T allele was predominant. In control subjects, the frequency of TT and CT genotypes was 99.3% and 0.7% respectively, while that of T and C alleles was 99.6% and 0.4% respectively. The frequency of CT genotype was higher in HCC (3.0%), while that of T and C alleles was 98.5% and 1.5%. Although the frequency of CT genotype and C allele was higher in HCC patients than in control subjects, but there was no significant difference.

The frequency of *DNMT3B* genotypes is much different from the reported frequency in Caucasians[21]. In the control group, the frequency of TT, CT and CC was 23.2%, 41.8% and 35.0% respectively, while the T allele frequency was 44.1%. C allele was very common. The frequency of TT, CT and CC in lung cancer patients was 21.0%, 56.7% and 22.3% respectively, indicating that a different race has a different genetic composition.

The *DNMT3B* allele frequency of people in North China is slightly different from that of people in other areas of China[30]. In the present study, the CC genotype was also not detectable. In control subjects, the frequency of TT and CT genotypes was 94.9% and 5.1% respectively, while that of T and C alleles was 97.4% and 2.6% respectively, suggesting that *DNMT3B* genetic polymorphism is variable in the same race living in different geographic areas. Only the TT genotype was detectable in all control subjects as compared with other ethnic groups such as Japanese[31], also suggesting that a different ethnic group has a different genetic composition.

The PCR product was digested with *Bln* I for 12-16 h to complete the reaction of restriction enzyme. Since the sensibility of electrophoresis through polyacrylamide gel is much higher than that of electrophoresis through agarose gel, we chose the former to separate the DNA fragments.

In our study, only one of the case subjects with CT genotype had a family history of HCC, but HBsAg, HBeAb and HBcAb in the other case subjects remained positive for a period of over 10 years without any treatment. Although we designed experiments to assess the correlation of the distribution of *DNMT3B* genotypes to the transcription and expression of *DNMT3B* and HBV infection status in selected tumor and normal hepatic tissues, we could not carry out the experiments due to the insufficient number of samples with CT genotype in our study.

The very similar distribution of *DNMT3B* genotypes in HCC patients and healthy controls suggested that the C/T polymorphism of *DNMT3B* gene might not independently affect the risk for HCC. A study in North China showed that *DNMT3B* SNPs are not associated with the susceptibility to gastric cardiac adenocarcinoma[30], although this polymorphism has been demonstrated to be associated with the susceptibility to cancers of the lung, head, neck and breast.

Recently, several candidate SNPs in the *DNMT3B* gene have been deposited in public databases (http://www.ncbi.nlm.nih.gov/SNP). Although the functional effects of these polymorphisms have not been elucidated, we hypothesize that some of these variants, particularly their haplotypes, may influence DNMT3B activity on DNA methylation, thereby modulating the susceptibility to HCC. The C/T SNP (C46359T) in the promoter of the *DNMT3B* gene may not be associated with up-regulation of DNMT3B and an increased risk of HCC as observed in this study, but probably there are other polymorphisms in *DNMT3B* which are susceptible to HCC.

In the present study, the *DNMT3B* gene was not negligible in the study of hepatocarcinogenesis in different areas. Whether *DNMT3B* SNPs are associated with different tumor types needs to be further studied in China. To clarify the role of *DNMT3B* SNP in the development of HCC, investigations in other populations need to be performed as well. The potential usefulness of *DNMT3B* genotyping needs further studies on a larger scale.

Saito[32] has found that over-expression of a splice variant of DNMT3B, DNMT3B4, which may lack DNA methyltransferase activity and compete with DNMT3B3 for targeting pericentromeric satellite regions, results in DNA hypomethylation on these regions, even at precancerous stages, and plays a critical role in the development of human HCC because of chromosomal instability and aberrant expression of cancer-related genes. Further investigation on the mechanism of aberrant expression of DNMT3B in tumors, including HCC, should be performed.

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COMMENTS

Background

Genetic and epigenetic factors are important in determining individuals' susceptibility to hepatocellular carcinoma (HCC). Alterations in DNA methylation can cause changes in gene transcription patterns and also promote mutational events leading to malignant tumors. DNA methylation is mediated by DNA methyltransferases (DNMTs), of which three active forms have been identified: DNMT1, DNMT3A and DNMT3B. DNMT3B, regarded as a *de novo* DNA methyltransferase, is thought to play an important role in the generation of aberrant methylation in carcinogenesis.

Research frontiers

Studies have shown that significant over-expression of DNMT3B is observed in tumor tissues while over-expression of DNMT1 and DNMT3A is more modest. A C-to-T transition polymorphism (C46359T) in the promoter region of the *DNMT3B* gene, -149 base pairs from the transcription start site, greatly increases promoter activity and is associated with an increased risk of cancers, such as cancer of the lung, head and neck. Carriers of the T allele, particularly heterozygotes, have a significantly increased risk foe such cancers.

Innovations and breakthroughs

Since the *DNMT3B* promoter polymorphism that is responsible for regulating genomic methylation is possibly associated with an increased risk for cancer, we evaluated the relationship between *DNMT3B* C46359T polymorphism and risk of HCC using PCR-RFLP and sequencing analysis.The PCR product was digested with *Bln* I for 12-16 h to complete the reaction of restriction enzyme. Since the sensibility of electrophoresis through polyacrylamide gel is much higher than that of electrophoresis through agarose gel, we chose the former to separate the DNA fragments.

Applications

C/T polymorphism of the *DNMT3B* gene may not independently affect the risk for HCC and probably there are other polymorphisms in *DNMT3B* which are susceptible to HCC. Whether *DNMT3B* SNPs are associated with different tumor types and different races needs to be further studied. The potential usefulness of *DNMT3B* genotyping needs further studies in a large scale.

Terminology

Single nucleotide polymorphisms (SNPs) represent a natural genetic variability at a high density in the human genome. A synonymous expression is "biallelic marker" corresponding to the two alleles that may differ in a given nucleotide position of diploid cells. A single SNP represents an alternative nucleotide in a given and defined genetic location at a frequency exceeding 1% in a given population. This definition does not include other types of genetic variability like insertions and deletions, and variability in copy number of repeated sequences. SNPs are considered the major genetic source to phenotypic variability that differentiates individuals from one another within a given species. Restriction fragment length polymorphism (RFLP) is a technique by which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of fragments produced differs when DNA is digested with a restriction enzyme. The similarity of patterns generated can be used to differentiate species (and even strains) from one another.

Peer review

Dr. Wu and co-investigators looked at C/T SNP in the promoter region of *DNMT3B*. They could not demonstrate a significant difference in C/T polymorphism between HCC patients and normal Chinese population. However, *DNMT3B* gene is not negligible in study of HCC in different races, ethnic groups or geographic areas as well as in study of different tumor types.

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CASE REPORT

Mycophenolate mofetil for drug-induced vanishing bile duct syndrome

S Simona Jakab, A Brian West, Dennis M Meighan, Robert S Brown Jr, William B Hale

S Simona Jakab, Dennis M Meighan, William B Hale, Section of Gastroenterology, Norwalk Hospital, Norwalk, CT 06856, United States
A Brian West, Department of Pathology, New York University, New York, NY, United States
Robert S Brown Jr, Center for Liver Disease and Transplantation, New York-Presbyterian Hospital, Columbia University College of Physicians and Surgeons, New York, NY, United States
Correspondence to: William B Hale, MD, Norwalk Hospital, Section of Gastroenterology, 24 Stevens Street, Norwalk, CT 06856, United States. william.hale@norwalkhealth.org
Telephone: +1-203-8522278 Fax: +1-203-8522075
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Abstract

Amoxicillin/clavulanate is associated with liver injury, mostly of a cholestatic pattern. While outcomes are usually benign, progression to cirrhosis and death has been reported. The role of immunosuppressive therapy for patients with a protracted course is unclear. We report the case of an elderly patient who developed prolonged cholestasis secondary to amoxicillin/clavulanate. Vanishing bile duct syndrome was confirmed by sequential liver biopsies. The patient responded to prednisone treatment, but could not be weaned off corticosteroids, even when azathioprine was added. Complete withdrawal of both prednisone and azathioprine was possible by using mycophenolate mofetil, an inosine monophosphate dehydrogenase inhibitor. Sustained remission has been maintained for more than 3 years with low-dose mycophenolate mofetil.

Key words: Amoxicillin and clavulanate; Drug-induced cholestasis; Ductopenia; Mycophenolate mofetil; Vanishing bile duct syndrome

Jakab SS, West AB, Meighan DM, Brown RS Jr, Hale WB. Mycophenolate mofetil for drug-induced vanishing bile duct syndrome. *World J Gastroenterol* 2007; 13(45): 6087-6089

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INTRODUCTION

Acute liver injury caused by amoxicillin/clavulanate occurs in 1.7 cases per 10000 prescriptions written and is mostly of a cholestatic type[1]. Outcomes are usually benign with resolution of cholestasis in 1-4 mo following drug withdrawal[2]. However, some patients develop prolonged drug-induced cholestasis, defined as the persistence of jaundice for more than 6 mo or persistently high alkaline phosphatase and gamma-glutamyl transpeptidase for more than 1 year, despite withdrawal of the causative drug, and in the absence of pre-existing liver or biliary tract disease[3]. Patients who develop progressive destruction of the small interlobular bile ducts ("vanishing bile duct syndrome") may ultimately require liver transplantation[4,5], given the lack of effective treatment. As an immunological reaction is suspected, corticosteroids have been used empirically[6], although the precise mechanism of amoxicillin/clavulanate-induced cholestatic hepatitis is unknown. We report a case illustrating that mycophenolate mofetil can be a successful and safe alternative to corticosteroids for amoxicillin/clavulanate-induced prolonged cholestasis.

CASE REPORT

A 69-year-old man presented with fatigue, upper abdominal discomfort and a pruritic rash involving his torso. He had a history of long-standing and well-controlled polycythemia vera. His medications included aspirin and hydroxyurea, and he drank alcohol sparingly. Three weeks prior to this examination, he had undergone a course of amoxicillin/clavulanate, 875 mg twice daily, for treatment of bronchitis. Physical examination revealed a fine maculopapular rash on his torso. The liver edge was palpable under the costal margin, and was smooth and not tender. No hepatosplenomegaly was noted. Initial laboratory data showed: alkaline phosphatase 624 U/L, aspartate aminotransferase (AST) 89 U/L, alanine aminotransferase (ALT) 82 U/L, total bilirubin 1.6 mg/dL, direct bilirubin 1.0 mg/dL, gamma glutamyl transpeptidase (GGT) 360 U/L, and albumin 3.3 g/dL. Abdominal ultrasound examination showed that the liver had a heterogeneous texture, with normal bile ducts and gallbladder. Over the next 2 weeks, he became jaundiced, with a peak of alkaline phosphatase of 988 U/L, total bilirubin 7.4 mg/dL, direct bilirubin 6.7 mg/dL, AST 235 U/L, and ALT 310 U/L. Prothrombin time (PT)/international normalized ratio (INR) remained normal. Autoimmune and viral serologic studies were all negative. Iron studies and alpha-1-antitrypsin levels were within normal ranges. Endoscopic retrograde cholangiography was normal. Liver biopsy, performed 5 mo after

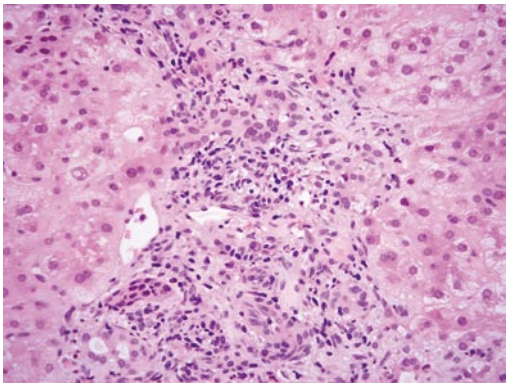

Figure 1 Initial biopsy. The triad is expanded by portal and periportal fibrosis, and there is a mixed inflammatory infiltrate. Injured bile ducts are present (bottom left and top center) and there is a marked ductular reaction.

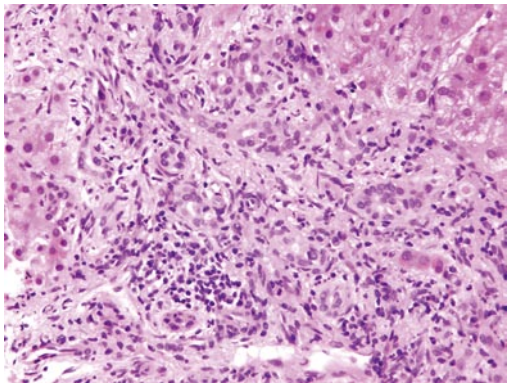

Figure 2 Second biopsy. The portal tracts appear similar to those seen in the initial biopsy, but with greater fibrous expansion ands more inflammation, including a loose aggregate of plasma cells and lymphocytes (bottom left). There is ductopenia and a prominent ductular reaction.

exposure to amoxicillin/clavulanate, showed destructive cholangiopathy of the small/medium-sized bile ducts, with ductopenia, duct proliferation, and bilirubinostasis. An infiltrate of lymphocytes and plasma cells was mostly confined to the portal tracts. Portal, periportal and focal bridging fibrosis were present, but hepatic architecture was preserved (Figure 1).

Treatment with prednisone 30 mg/d and ursodiol 1200 mg/d resulted in marked clinical and biochemical improvements. Tapering off the prednisone dose was attempted over the next few months, but resulted in increased alkaline phosphatase, AST and ALT, every time the prednisone dose was decreased to 12 mg/d. Azathioprine, titrated to 100 mg/d for several months, could not achieve prednisone tapering.

A second liver biopsy, taken after more than 1 year of treatment, showed chronic destructive cholangiopathy, persistent portal inflammation, and progression of fibrosis, with portoportal bridging and distorted hepatic architecture (Figure 2). Repeat endoscopic retrograde cholangiopancreatography (ERCP) revealed no extrahepatic biliary abnormalities.

Mycophenolate mofetil was added at a dose of 1 g twice daily and the patient was rapidly weaned off azathioprine. Over the next 6 mo, prednisone was successfully tapered, with liver tests remaining normal, except for a mild elevation in alkaline phosphatase (Figure 3). Over 1 year, the dose of mycophenolate mofetil was reduced to 250 mg twice daily, without adverse effects. Efforts to withdraw treatment completely resulted in recurrence of mild cholestatic abnormalities.

DISCUSSION

About 30 drugs, including amoxicillin/clavulanate, have been reported to cause vanishing bile duct syndrome with protracted clinical courses, the prototype being chlorpromazine[4]. Ductopenia, when interlobular bile ducts are absent from at least 50% of the small portal tracts, carries a poor prognosis[7]. The mechanism of progression from acute liver injury to ductopenia is unclear. However, it is suggested that bile ducts, as complete epithelium-lined tubes, are only rarely reconstructed once they have been completely destroyed[8]. A patient's unique immune response likely plays a role in the intensity and duration of injury, as certain HLA haplotypes have been found to be markedly overrepresented in patients who develop drug-induced cholestatic hepatitis[9,10].

Our case had the typical characteristics of amoxicillin/clavulanate-induced liver injury. These include advanced age, male sex, a cholestatic pattern of liver injury, delay between cessation of therapy and onset of jaundice, repeatedly negative tests for viral, autoimmune and metabolic diseases, and negative imaging studies[2,11-14]. Primary biliary cirrhosis and autoimmune cholangiopathy were considered unlikely, given the patient's age, gender, repeatedly negative serology, and histopathology.

A distinctive feature of our patient was his first liver biopsy that showed destructive cholangiopathy with portal and periportal fibrosis. The second biopsy, obtained 1 year later, revealed persistence of the destructive cholangiopathy but worsening fibrosis, with portoportal bridging and architectural distortion. In our patient, early fibrosis at 5 mo after exposure to amoxicillin/clavulanate may explain the prolonged cholestasis and immunosuppressant dependency. Patients reported complete recovery from prolonged amoxicillin/clavulanate-induced cholestasis did not have fibrosis on liver biopsy[2,11-14]. By contrast, in Degott's review of drug-induced cholestasis, all patients with persistent cholestasis had moderate to severe fibrosis[15].

Given the small number of patients reported with drug-induced vanishing bile duct syndrome and the unpredictability of its occurrence, there have been no clinical trials of treatment regimens. Short courses of corticosteroids have been used[6], based on a suggested immune pathogenesis of drug-induced cholestatic hepatitis. Mycophenolate mofetil, a non-competitive inhibitor of purine synthesis that acts by inhibiting inosine monophosphate dehydrogenase, blocks T- and B-lymphocyte proliferation. Approved for prophylaxis of rejection in solid organ transplantation, it is used against various immune-mediated diseases. Small clinical trials have reported its efficacy as a corticosteroid-sparing agent in autoimmune hepatitis[16,17], but it has not been evaluated for use in drug-induced liver disease. Our case illustrates

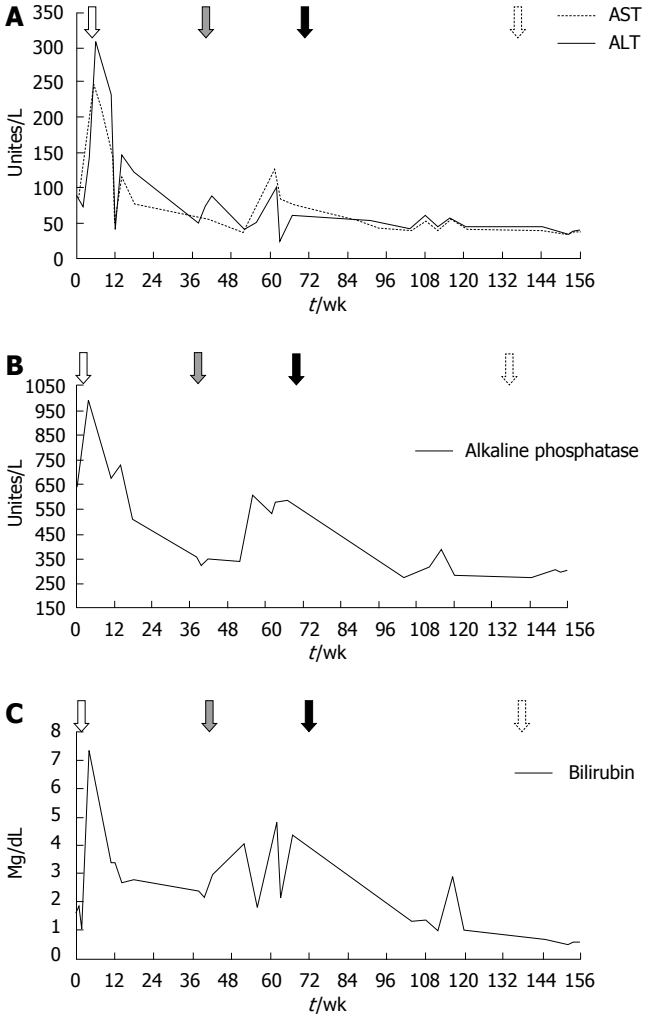

Figure 3 Three-year follow-up: liver enzymes and bilirubin during treatment with prednisone (white arrow), azathioprine/prednisone (grey arrow), and mycophenolate mofetil/prednisone (black arrow). Prednisone was stopped at wk 140 (dashed arrow). **A**: AST/ALT; **B**: alkaline phosphatase; **C**: bilirubin.

that mycophenolate mofetil can be a successful and safe alternative to corticosteroids for drug-induced prolonged cholestasis. Our patient has, at the time of this report, maintained normal liver function for over 3 years on low-dose mycophenolate mofetil, though efforts to withdraw treatment completely resulted in recurrence of mild cholestatic abnormalities.

In summary, we reported a case of severe amoxicillin/clavulanate-induced cholestatic hepatitis that resulted in progressive bile duct destruction and development of bridging fibrosis. Clinical and biochemical resolution was achieved using long-term immunosuppression, initially with prednisone and finally with low-dose mycophenolate mofetil. This suggests that cautious use of immunosuppressive therapy may be of benefit in those rare cases with persistent cholestasis. Further studies are needed to determine if early therapy can prevent irreversible bile duct injury, and to identify patients in whom such therapy is indicated.

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CASE REPORT

Sirolimus-induced drug fever and ciclosporin-induced leukencephalopathia with seizures in one liver transplant recipient

Doris Schacherer, Martina Zeitoun, Roland Büttner, Cornelia Gelbmann, Aiman Obed, Hans-Jürgen Schlitt, Jürgen Schölmerich, Gabi I Kirchner

Doris Schacherer, Martina Zeitoun, Roland Büttner, Cornelia Gelbmann, Jürgen Schölmerich, Gabi I Kirchner, Department of Internal Medicine I , University of Regensburg, Regensburg 93042, Germany
Aiman Obed, Hans-Jürgen Schlitt, Department of Surgery, University of Regensburg, Regensburg 93042, Germany
Correspondence to: Dr. Doris Schacherer, Klinik und Poliklinik für Innere Medizin I , Universitätsklinikum, Regensburg 93042, Germany. doris.schacherer@klinik.uni-regensburg.de
Telephone: +49-941-9447001 Fax: +49-941-9447002
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Abstract

We describe the first case of sirolimus-induced drug fever in a female liver transplant recipient, with a history of hepatitis C-induced end-stage liver cirrhosis in 1999. In 2005, six years after transplantation, she developed calcineurin inhibitor-induced renal function impairment. Immunosuppression was switched from tacrolimus to sirolimus. Two days after the intake of sirolimus, she developed daily fever spikes, but no infectious focus was found. Antibiotic therapy had no influence on the fever. After fourteen days, sirolimus was switched back to tacrolimus and the fever disappeared. In history, the patient developed ciclosporin-induced generalized seizures eleven days after liver transplantation, followed by the development of a motoric speech disorder. Magnetic resonance imaging (MRI) findings were consistent with leucoencephalopathy, therefore immunosuppressive therapy was changed from ciclosporin to tacrolimus and the neurologic symptoms improved significantly. Our case is the first reported case of sirolimus-induced drug fever. In addition, the patient showed the rare occurrence of ciclosporin-induced leukencephalopathy with seizures.

Key words: Liver transplantation; Immunosuppression; Side effects

Schacherer D, Zeitoun M, Büttner R, Gelbmann C, Obed A, Schlitt HJ, Schölmerich J, Kirchner GI. Sirolimus-induced drug fever and ciclosporin-induced leukencephalopathia with seizures in one liver transplant recipient. *World J Gastroenterol* 2007; 13(45): 6090-6093

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INTRODUCTION

Ciclosporin and tacrolimus are very potent immunosuppressive drugs which have been used in organ transplantation for more than 10 years. Both are calcineurin-inhibitors and have the same mode of action. They are mainly metabolized by cytochrome P450 3A4 in bowel and liver[1]. The main side effects of ciclosporin and tacrolimus are renal toxicity, neurotoxicity, arterial hypertension, diabetes mellitus and hyperlipidemia[2]. Combination therapy of these two drugs is therefore not useful. Until now, many immunosuppressive regimens contain either ciclosporin or tacrolimus in combination with other immunosuppressive drugs. For several years, the mammilian target of rapamycin (mTOR) inhibitor, sirolimus (Rapamune®), has also been used as an immunosuppressive drug in organ transplantation. Similar to ciclosporin, it is also metabolized in bowel and liver by cytochrome P450 3A[1]. The main advantage of sirolimus is its virtual lack of nephrotoxicity. The main side effects are hyperlipidemia, anemia, and thrombocytopenia. Ciclosporin and sirolimus are substrates of the efflux-transporting pump P-glycoprotein, which is among others localized in gut and liver[3]. Ciclosporin and sirolimus have different modes of action and synergistic effects[4]. Therefore, in patients with renal impairment, combination therapy allows a dose reduction of both drugs which results in reduced side effects, especially on renal toxicity. Ciclosporin, tacrolimus as well as sirolimus can be given as monotherapy or in combination with other drugs. Patients with progressive impairment in renal function due to ciclosporin- or tacrolimus-induced nephrotoxicity can be switched to sirolimus monotherapy to prevent further loss of renal function[5].

CASE REPORT

We present the case of a 63-year-old female patient, who was first diagnosed with chronic hepatitis C virus infection in 1996 (genotype 1b). Infection was most likely due to several blood transfusions which were necessary after resection of a cyst of the left ovary in 1973. In 1997

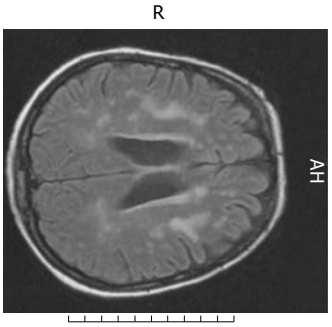

Figure 1 Magnetic resonance imaging (MRI) of the head showing almost symmetric periventricular white matter lesions consistent with leucoencephalopathy and a beginning encephalitis (FLAIR TR 8000 ms, TE 110 ms).

histopathological liver examination was performed for the first time and showed inflammation grade Ⅳ, as well as fibrosis grade Ⅲ (Ishaak-Score). Therefore, interferon monotherapy was given for one year (relapse after end of treatment). Six months later, she presented with decompensated liver cirrhosis and esophageal bleeding due to varices grade Ⅲ. After re-compensation she was listed for liver transplantation in January 1999. Neurologic status was normal prior to liver transplantation. Liver transplantation was successfully performed in August 1999. The explanted liver demonstrated complete cirrhosis (716 g). The liver graft showed no histological damage and normal perfusion as judged by duplex sonography. Immunosuppressive therapy was started with a combination therapy of ciclosporin, azathioprine and steroids. Eleven days after the start of immunosuppressive therapy, she developed a generalized seizure, which could be stopped with diazepame. Several focal and generalized epileptic fits followed and the patient developed a motoric speech disorder which finally resulted in dysarthria and complete aphasia. The patient showed no other neurologic symptoms. A magnetic resonance imaging (MRI) of the head showed periventricular white matter lesions consistent with leucoencephalopathy and a beginning encephalitis (Figure 1). The described symptoms were interpreted as side effects of ciclosporin medication. Therefore, immunosuppressive therapy was changed from ciclosporin to tacrolimus (FK 506, Prograf®) and antiepileptic therapy was initiated with 400 mg gabapentin four-times a day. The dysarthric disorder improved significantly, but residuals still existed with no occurrence of further seizures at the time when this report was written. During the treatment with steroids and tacrolimus, the patient developed an insulin-dependent diabetes mellitus.

In December 1999, transplant reinfection with hepatitis C virus was diagnosed. In July 2001, liver histology showed a beginning fibrosis of the liver graft with little infiltration of inflammatory cells, corresponding to chronic hepatitis (Yano classification: grading 4, staging 3). Therefore, antiviral therapy with interferon alpha (1.5 Mio units/wk) and ribavirin (600 mg/d) was initiated. Due to pancytopenia azathioprine medication, ribavirin was stopped one year later. Hepatitis C virus re-infection was controlled by 90 μg pegylated interferon alpha 2a once a week subcutaneously. In 2003, a control MRI showed no deterioration of leukencephalopathy.

In September 2005, the patient presented with progressive renal impairment and peripheral oedema. Serum creatinine (193 μmol/L, normal range 44-80 μmol/L) and urea levels (15 mmol/L, normal range 2.0-8.3 mmol/L) were elevated. Urinary tests confirmed chronic renal failure with a creatinine clearance of only 20 mL/min. Therefore, immunosuppressive therapy was changed from tacrolimus (Prograf®) to sirolimus (Rapamune®). Two days after starting sirolimus, the patient developed fever of 38℃, which reached up to 39℃ on the next day. No reason for the fever was found either with blood cultures or with urine examination, or with X-rays. The patient showed no other specific clinical symptoms (e.g. diarrhea, skin lesions, pharyngitis). Leucocytes (3.8/nL, normal range 4.8-10.8/nL) and thrombocytes (96/nL; normal range 130-440/nL) were reduced, most likely due to interferon therapy and splenomegaly. Erythrocyte sedimentation rate (ESR) and C-reactive protein were normal and cytomegalovirus and Epstein Barr virus screening was negative. Antibiotic therapy with piperacilline and sulbactame was given for 6 d, but did not result in any improvement of the daily spikes of fever, which occurred every evening (Figure 2). After 14 d, the fever disappeared when sirolimus was switched back to tacrolimus in combination with mycophenolate mofetil. Thus, sirolimus-induced drug fever was diagnosed.

At the time when we wrote this report, the patient was in a good clinical condition, neurologic status remained stable but dysarthria still existed, and renal function (133 μmol/L serum creatinine) improved with dose reduction of tacrolimus and intake of 3 litres fluid per day.

DISCUSSION

The clinical and radiological features demonstrated in this patient are consistent with those of leukoencephalopathy, a rare condition previously described in patients treated with ciclosporin and tacrolimus[6,7]. In our patient as in most patients previously described, leukoencephalopathy associated with immunosuppression occurred early during therapy and was reversible with good recovery. One case of late-onset leukoencephalopathy with a fatal outcome has been reported[8]. In the reported case, the symptoms due to leukoencephalopathy improved after withdrawal of ciclosporin, but unfortunately, did not completely disappear. The patient also suffered from seizures, which disappeared after withdrawal of ciclosporin and initiation of antiepileptic therapy with gabapentin.

Neurologic symptoms represent serious complications following orthotopic liver transplantation and may be caused by various perioperative factors or may develop due to drug-specific toxicity of immunosuppression. The incidence of neurotoxicity seems to be higher in patients treated with tacrolimus than in patients treated with ciclosporin in the early postoperative period, after retransplantation as well as in the late phase[9]. Watson *et al*[10] described two patients who suffered from neurological events, one with encephalopathy and the other with recurrent seizures. Both patients were on sirolimus and ciclosporin after orthotopic liver transplantation and stabilized after withdrawal of ciclosporin. Choi *et al*[11] reported that of the 367 patients who received OLT, 48 suffered from neurological complications, 17

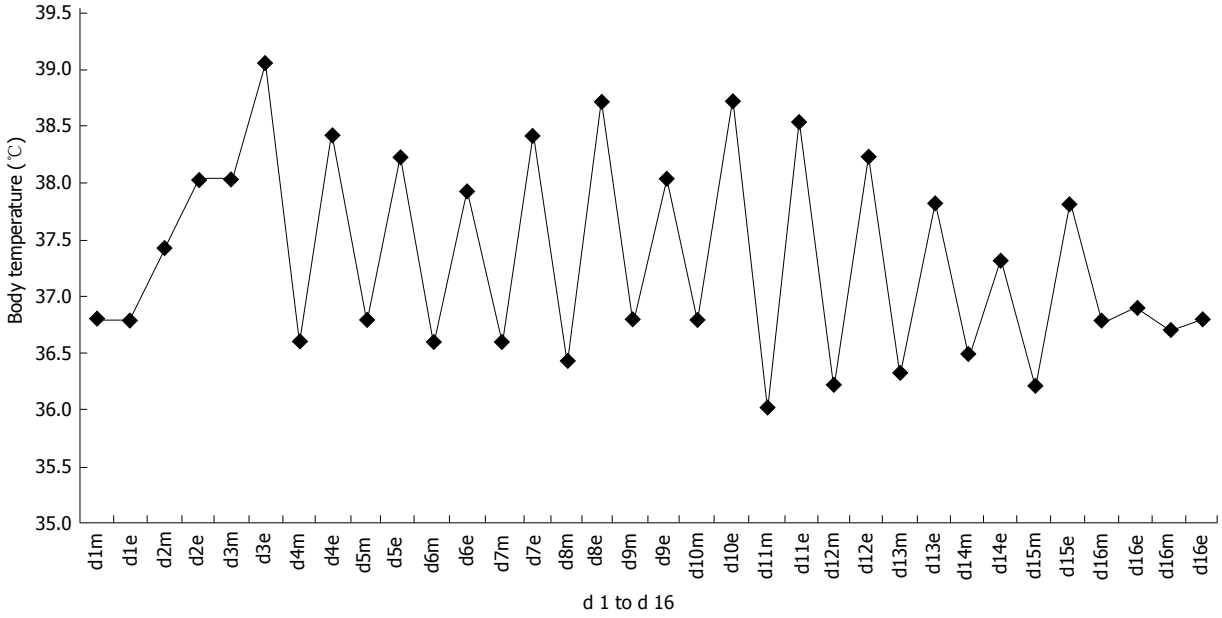

Figure 2 Temperature curve before, during (14 d) and after sirolimus therapy. Antibiotic therapy with piperacilline and sulbactame given from d 3 to 9 showing no effect. m: morning; e: evening.

developed seizures (status epilepticus occurred in two patients, generalized tonic-clonic seizures in five patients). Although neurotoxicity is not a frequent side effect of ciclosporin medication, the described cases in the reports are in accordance with our patient's symptoms (seizures and consecutive motoric speech disorder) which could be interpreted as side effects of ciclosporin medication. The motoric dysarthric disorder improved significantly after a change of the immunosuppressive regimen, but residuals still existed.

Idilman *et al*[12] described two cases of reversible posterior leukoencephalopathy manifested as headache, nausea and seizures associated with the use of immunosuppressive drugs following liver transplantation. One case of a 29-year old patient treated with ciclosporin after a liver transplant for primary sclerosing cholangitis showed late-onset progressive leucoencephalopathy due to immunosuppressive therapy and died three years later. These reports suggest that neurological side effects should be cautiously observed when alteration of immunosuppressive therapy is considered.

Although ciclosporin is an immunosuppressive agent widely used in the management of liver transplant recipients, neurological complications have been described in only few cases. The two different neurological side effects found in our patient are probably associated with ciclosporin medication.

Side effects of sirolimus include delayed wound healing, oral ulcers, hypertension, interstitial pneumonitis, infections, and most importantly, hyperlipidemia and myelosuppression[13]. Concerning the central nervous system, it has been shown that sirolimus can alter cell metabolism of primary astrocytes, thus resulting in similar neurotoxicity as experienced by tacrolimus and ciclosporin[14]. Perhaps the greatest potential benefit of sirolimus for liver transplant recipients is its lack of nephrotoxicity as compared to calcineurin inhibitors[15,16]. At present, no single immunosuppressive regimen can offer a clear advantage over another with regard to prevention of cellular rejection, graft survival, and patient survival[17]. In our patient, immunsuppression was switched from ciclosporin to sirolimus due to nephrotoxicity of ciclosporin. We clearly could show that the fever in our patient was not related to infection, but most likely to sirolimus. Two days after starting immunosuppression with sirolimus, our patient developed fever with no infectious focus found in blood cultures, urine tests or in radiologic examinations. Even the antibiotic therapy did not show any improvement of the daily spikes of fever in the evening. Due to the diagnosis of sirolimus-induced drug fever, the immunosuppressive medication was changed back to tacrolimus in combination with mycophenolate mofetil and no more fever spikes occured. To our knowledge, this is the first reported case of drug-fever obviously related to sirolimus. Two years ago, Dorschner *et al*[18] described a 2-year drug-related fever caused by everolimus, a sirolimus-derived immunosuppressant (Certican®). Their patient was 66 years old and received a cardiac transplant due to dilatative cardiomyopathy. The immunosuppressive regimen consisted of steroids, ciclosporin, and everolimus. Two weeks after the replacement of everolimus with azathioprine, all the patient's symptoms disappeared.

This is the first report of a liver transplant recipient with rare immunosuppressant-induced side effects. Until now, we could not unravel the mechanism(s) responsible for these side effects. A mutation in cytochrome P450 3A or FK-binding protein 12 (FK-BP12) seems to be unlikely, because our patient had no side effects under medication with tacrolimus, which is also metabolised by cytochrome P450 3A and bound to FK-BP12. Since ciclosporin, tacrolimus and sirolimus are substrates of the ATP-binding efflux pump P-glycoprotein, located in several organs like bowel and liver, it seems to be impossible that this protein might cause the several drug-side effects in our

patient. Therefore, we speculate that immunosuppressant drugs may have some influence on proteins in the central nervous system.

In conclusion, ciclosporin is an immunosuppressive agent widely used in the management of solid organ transplantation. Sirolimus is a powerful immunosuppressant used to prevent acute rejection episodes in patients who have undergone transplantation, particularly when nephrotoxic effects of calcineurin inhibitors become problematic.

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CASE REPORT

Carcinoembryonic antigen-producing adrenal adenoma resected using combined lateral and anterior transperitoneal laparoscopic surgery

Tomohide Hori, Kentaro Taniguchi, Masashi Kurata, Kenji Nakamura, Kenji Kato, Yoshifumi Ogura, Makoto Iwasaki, Shinya Okamoto, Koichiro Yamakado, Shintaro Yagi, Taku Iida, Takuma Kato, Kanako Saito, Linan Wang, Yoshifumi Kawarada, Shinji Uemoto

Tomohide Hori, Yoshifumi Kawarada, Department of Hepatobiliary Pancreatic Surgery, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Kentaro Tanoguchi, Kenji Nakamura, Kenji Kato, Yoshifumi Ogura, Department of Surgery, Matsusaka City Hospital, 1550 Tono-machi, Matsusaka City, Mie Prefecture, 515-8544, Japan
Masashi Kurata, Department of Anesthesiology, Matsusaka City Hospital, 1550 Tono-machi, Matsusaka City, Mie Prefecture, 515-8544, Japan
Makoto Iwasaki, Department of Surgery, Iwasaki Hospital, 333 Isshinden-cho, Tsu City, Mie Prefecture, 514-0114, Japan
Shinya Okamoto, Department of Cardiology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Koichiro Yamakado, Department of Radiology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Shintaro Yagi, Taku Iida, Shinji Uemoto, Department of Hepatobiliary Pancreatic and Transplant Surgery, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
Takuma Kato, Linan Wang, Department of Cellular and Molecular Immunology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Kanako Saito, Department of Medical Oncology and Immunology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan
Correspondence to: Tomohide Hori, Department of Hepatobiliary Pancreatic Surgery and Breast Surgery, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie Prefecture, 514-8507, Japan. tomohide@clin.medic.mie-u.ac.jp
Telephone: +81-59-2321111 Fax: +81-59-2328095
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Abstract

A 74-year-old woman presented with symptoms consistent with hyperadrenocorticism and hypercatecholaminism. She had a cushingoid appearance and her cortisol level was elevated. Her serum dopamine and noradrenalin levels were also elevated. Computed tomography detected a left adrenal mass measuring 3.5 cm × 3.0 cm in diameter. Metaiodobenzylguanidine scintigraphy was negative. Unexpectedly, the serum Serum carcinoembryonic antigen (CEA) level was elevated. Fluorodeoxyglucose positron emission tomography showed increased uptake in the adrenal tumor only, with a maximum standardized uptake value of 2.8. Selective venography and blood sampling revealed that the concentrations of cortisol, catecholamines and CEA were significantly elevated in the vein draining the tumor. A diagnosis of CEA-producing benign adenoma was made. After preoperative management, we performed a combined lateral and anterior transperitoneal laparoscopic adrenectomy. Her vital signs remained stable during surgery. Histopathological examination revealed a benign adenoma. Her cortisol, catecholamine and CEA levels normalized immediately after surgery. We present, to the best of our knowledge, the first case of CEA-producing adrenal adenoma, along with a review of the relevant literature, and discuss our laparoscopic surgery techniques.

Key words: Carcinoembryonic antigen; Laparoscopy; Adenoma; Adrenal gland; Cushing syndrome

Hori T, Taniguchi K, Kurata M, Nakamura K, Kato K, Ogura Y, Iwasaki M, Okamoto S, Yamakado K, Yagi S, Iida T, Kato T, Saito K, Wang L, Kawarada Y, Uemoto S. Carcinoembryonic antigen-producing adrenal adenoma resected using combined lateral and anterior transperitoneal laparoscopic surgery. *World J Gastroenterol* 2007; 13(45): 6094-6097

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INTRODUCTION

Serum carcinoembryonic antigen (CEA) level is widely used as a reliable tumor marker in cancer patients. CEA level is rarely elevated in benign disease, and to the best of our knowledge there have been no reports of CEA-producing benign adrenal tumors. We present the first reported case of a CEA-producing adrenal adenoma, along with a review of the relevant literature, and discuss a useful technique for the removal of adrenal tumors.

CASE REPORT

A 74-year-old Japanese woman was referred to our hospital

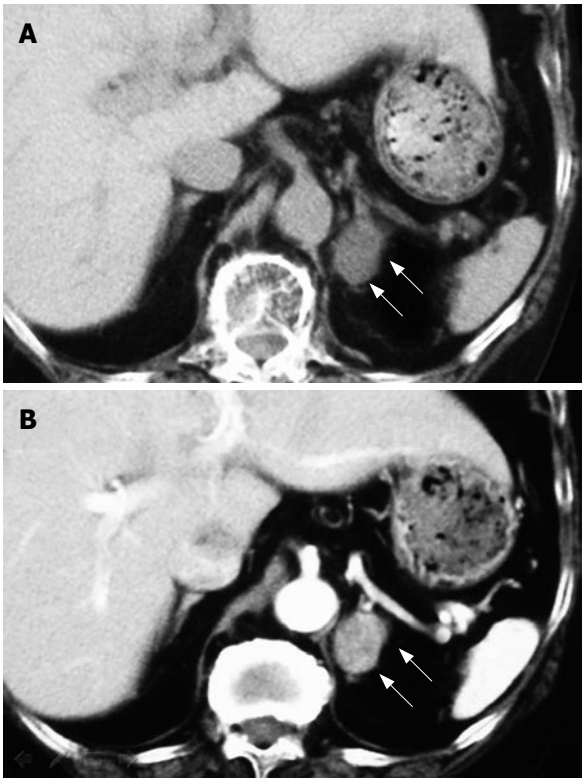

Figure 1 Abdominal CT. Plain (**A**) and enhanced (**B**) CT revealed a left adrenal mass (white arrows) measuring 3.5 cm × 3.0 cm in diameter.

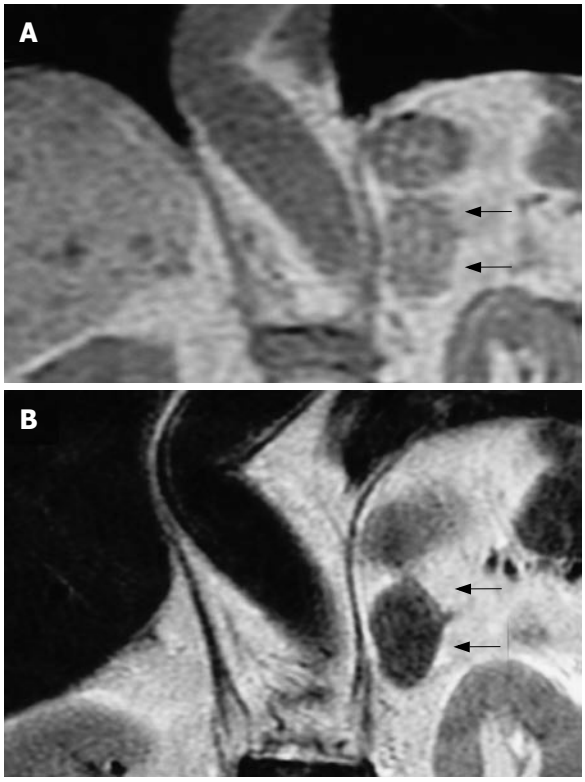

Figure 2 Abdominal MRI. MRI showed an adrenal mass (black arrow) with slightly low intensity on T1-weighted images (**A**) and low intensity on T2-weighted images (**B**).

because of fatigue and frequent episodes of palpitations, sweating, headache and weight gain over the past year. She had a history of hypertension and hyperglycemia, for which she had been treated at another hospital for 18 years. Although the number of medications and their dosages had slowly increased during the year before this consultation, her diseases and symptoms were not well controlled. Physical examination showed truncal obesity, moon face, buffalo hump and proximal muscle weakness. On admission, her blood pressure was 202/108 mmHg, and pulse rate 96 beats/min. X-ray images revealed osteoporosis. Serum biochemistry showed elevated levels of glucose (243 mg/dL; normal, 70-109 mg/dL), Hemoglobin A1c (HbA1c) (8.9%; normal, 4.3-5.8%) and total cholesterol (271 mg/dL; normal, 50-149 mg/dL), and reduced levels of total protein (5.5 g/dL; normal, 6.5-8.3 g/dL) and albumin (3.2 g/dL; normal, 3.7-5.3 g/dL). Abdominal computed tomography (CT) showed a left adrenal mass measuring 3.5 cm × 3.0 cm in diameter (Figure 1). Magnetic resonance imaging (MRI) showed an adrenal mass with slightly low intensity on T1-weighted images and low intensity on T2-weighted images (Figure 2). Her physical appearance was cushingoid, as described above. Adrenal cortical hormone analysis revealed a markedly elevated cortisol level, but aldosterone and estradiol levels were normal. The regulatory factors for these hormones were all within normal ranges (Table 1). Catecholamine analysis revealed markedly elevated dopamine and noradrenalin (NA) levels but the adrenalin level was within the normal range. The levels of catecholamine-breakdown products homovanillic acid (HVA) and vanillylmandelic acid (VMA) were both increased (Table 1). ^{123}I-metaiodobenzylguanidine scintigraphy showed no adrenal or extra-adrenal hot spots.

Unexpectedly, the CEA level was also found to be elevated (Table 1), although other tumor markers including carbohydrate antigen (CA) 19-9, CA125, CA15-3, alpha-fetoprotein and squamous cell carcinoma antigen were all normal. Upper gastrointestinal and colorectal endoscopy, neck/chest CT, and genital and breast examination did not reveal any abnormal findings. F-18 fluorodeoxyglucose (FDG)-positron emission tomography (PET)/CT revealed increased FDG uptake in the left adrenal gland (Figure 3). The maximum standardized uptake value was 2.8, and there was no FDG uptake elsewhere. We performed selective venography and blood sampling at various locations around the inferior vena cava to measure the concentrations of factors not within a normal range. The left adrenal vein (AV) was the drainage vein of the tumor and flowed into the left renal vein (RV). Results from the selective blood sampling clearly showed that the concentrations of these factors were dramatically increased in the left adrenal 'drainage' vein. This revealed that the left adrenal tumor was an obvious source of the CEA (Figure 4).

We diagnosed the tumor as a benign adrenal adenoma, which caused hyperadrenocorticism and hypercatecholaminism[1] and produced a large amount of CEA. After preoperative management[2], we removed the tumor by combined lateral and anterior transperitoneal laparoscopic adrenectomy (LA). The patient was placed in the right lateral position. A trocar was inserted at the umbilicus, and a carbon dioxide pneumoperitoneum (10 mmHg) was established. We introduced one trocar into the abdominal cavity through the lateral abdominal

Table 1 Time course of serum endocrine profiles and CEA levels before and after surgery

	Normal range	Before surgury	Postoperative day			
			1	3	5	7
Adrenal hormones						
Cortical secretion						
Hormones						
Aldosterone (pg/mL)	30.0-160.0	30.4	50.3	113.5	43.3	72.7
Cortisol (μg/dL)	5.0-15.0	54.2	11.2	9.9	10.6	7.2
Estradiol (pg/mL)	10.0-20.0	12.2	10.8	18.6	17.8	11.7
Regulatory factors for cortical hormones						
Adrenocorticotrophic hormone (pg/mL)	5.0-52.0	5.2	5.4	9.2	5.8	14.8
Angiotensin (pg/mL)	9.0-47.0	12.3	24.6	33.1	36.6	24.9
Renin (pg/mL)	2.5-21.4	3.7	13.9	16.1	8.9	17.6
Medullary secretion						
Catecholamines						
Dopamine (pg/mL)	< 14.0	15.1	6.5	8.5	6.2	11.5
NA (pg/mL)	46.0-60.0	102.7	52.7	47.1	50.9	59.1
Adrenalin (pg/mL)	< 70.0	40.0	45.8	65.2	64.6	61.2
Catecholamine breakdown products						
HVA (ng/mL)	4.0-7.8	8.8	4.5	4.2	5.2	6.5
VMA (ng/mL)	3.3-8.6	24.5	7.8	7.5	7.7	8.1
Tumor marker						
CEA (ng/mL)	< 5.0	12.6	4.5	2.5	2.1	2.2

Values outside the normal range are underlined.

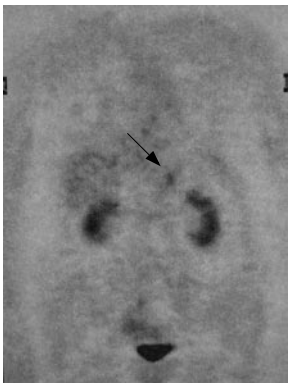

Figure 3 FDG-PET/CT. FDG uptake in the left adrenal gland (black arrow). The maximum standardized uptake value was 2.8, and no FDG uptake was seen anywhere else.

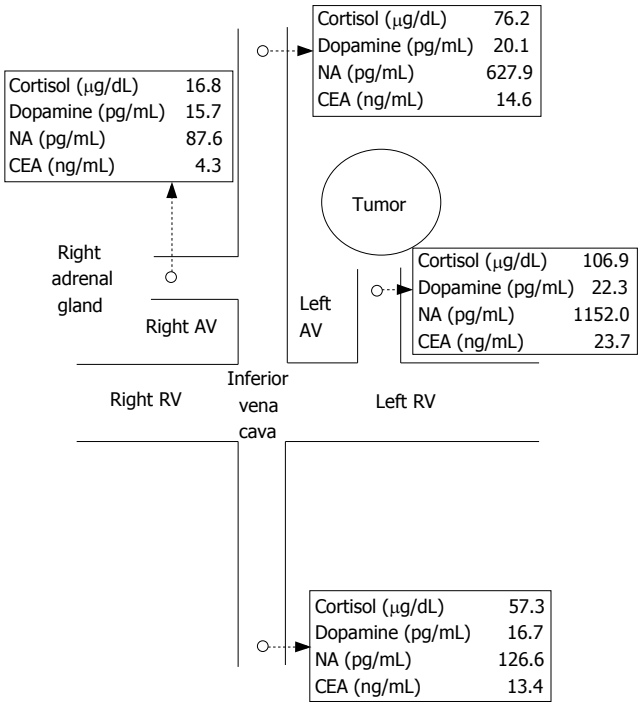

Figure 4 Selective venography and direct blood sampling. Measurements of serum cortisol, dopamine, NA and CEA were performed at four locations (proximal and distal inferior vena cava and both AVs). White circles represent sampling points.

wall, and two trocars through the subcostal wall. Using an electrothermal vessel sealing system (LigaSure, Tyco Healthcare, CO, USA), we isolated the spleen and distal pancreas by resecting the phrenic, colic and renal ligaments. The left RV and splenic vein were exposed by retracting the distal pancreas, spleen and left kidney. The left AV was separated from the surrounding tissue and ligated at its entry into the RV using a clip. The tumor was found to be located as indicated on preoperative imaging studies, without adhesion to the adjacent organ, and was removed with complete hemostasis. Vital signs remained stable during LA. Operative time was 2 h 45 m, and total blood loss was 85 mL. Histopathological examination of the resected specimen confirmed a benign adenoma. Chromaffin staining was negative. Cortisol, catecholamine and CEA levels normalized immediately after surgery (Table 1).

DISCUSSION

Based on hormonal evaluations of the adrenal cortex, we considered that this patient's hyperadrenocorticism was adrenocorticotrophic hormone-independent and renin-angiotensin-system-independent. Phenylethanolamine N-methyltransferase (PNMT), which is found in the adrenal glands, is required for the conversion of NA to adrenalin during catecholamine synthesis[3]. Catecholamine metabolism produces the breakdown products HVA (from dopamine) and VMA (from NA). Hormonal evaluations of the adrenal medulla suggested that this adrenal

adenoma either lacked PNMT or the optimal environment for the activation of PNMT.

LA was first performed in 1992[4,5], and this safe and effective treatment is now used worldwide for the management of functioning and non-functioning adrenal tumors for many reasons[6]. The minimal skin incisions provide a sufficient surgical field, anastomosis and reconstruction are not required, hemostasis can be achieved using laparoscopic devises, and the resected tumor can be removed through the small skin incision. Many previous reports on LA have already described the advantages and shortcomings of the transperitoneal and retroperitoneal approaches. There are two transperitoneal approaches, lateral and anterior[7]. Especially in left adrenal tumors and cases with more retroperitoneal fat, we have a clear impression that the combined lateral and anterior transperitoneal LA has the advantage of providing a sufficient surgical field and anatomical orientation in a timely manner. Surgery for catecholamine-releasing tumors differs from that for non-functioning tumors, because of the risk of intraoperative hypertensive and tachycardiac events[8]. In the present case, the combined approach allowed easy and early ligation of the drainage vein, which was the source of the catecholamines. We suggest that this procedure is effective for avoiding intraoperative iatrogenic complications.

CEA, the first tumor-associated antigen to be described, has many features that make it attractive for active vaccination against cancer. This useful biomarker is expressed in > 50% of all human cancers[9], including colorectal, lung, stomach, breast, pancreas, gallbladder, biliary tract, cervix, uterus, ovary, head/neck, bladder, kidney and prostate cancer. In the present study, we performed FDG-PET/CT to rule out adrenal cancer and to search for extra-adrenal tumors to explain the raised CEA level, as the conventional investigations for evaluation of an elevated CEA level showed no significant findings. We eventually hypothesized that the left adrenal tumor was producing a large amount of CEA, and performed selective venography and direct blood sampling of the drainage vein of the tumor to verify this. This method was useful for detecting the source of CEA in the present case. Previous reports have demonstrated that smoking, inflammatory diseases and benign tumors uncommonly produce CEA; but that benign tumors rarely progress to become malignant[10,11]. We are unable to explain how this patient's benign adenoma acquired the ability to secrete CEA, even after reviewing the relevant literature. This appears to be the first reported case of a CEA-producing adrenal adenoma. As a benign adrenal adenoma rarely secretes CEA, more cases need to be studied to better understand CEA production in benign adrenal tumors.

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CASE REPORT

Heterotopic pancreas in the stomach: A case report and literature review

Grigorios Christodoulidis, Dimitris Zacharoulis, Sotiris Barbanis, Emmanuel Katsogridakis, Konstantine Hatzitheofilou

Grigorios Christodoulidis, Dimitris Zacharoulis, Sotiris Barbanis, Emmanuel Katsogridakis, Konstantine Hatzi-theofilou, Department of Surgery, University Hospital of Larissa, Larissa 41221, Greece
Correspondence to: Grigorios Christodoulidis, MD, Department of Surgery, University Hospital of Larissa, Nikotsara 6, Larissa 41223, Greece. gregsurg@yahoo.gr
Telephone: +30-24-10239547 Fax: +30-24-10681170
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Abstract

Ectopic pancreas is defined as pancreatic tissue found outside the usual anatomic location of the pancreas. It is often an incidental finding and can be found at different sites in the gastrointestinal tract. It may become clinically evident when complicated by pathologic changes such as inflammation, bleeding, obstruction, and malignant transformation. In this report, a 40 years old woman with epigastric pain due to ectopic pancreatic tissue in the stomach is described. The difficulty of making an accurate diagnosis is highlighted. The patient has remained free of symptoms since she underwent wedge resection of the lesion three years ago. Frozen sections may help in deciding the extent of resection intraoperatively. Although ectopic pancreas is rare, it should be considered in the differential diagnosis of a submucosal gastric tumour.

Key words: Ectopic pancreas; Stomach; Histology; Surgery

Christodoulidis G, Zacharoulis D, Barbanis S, Katsogridakis E, Hatzitheofilou K. Heterotopic pancreas in the stomach: A case report and literature review. *World J Gastroenterol* 2007; 13(45): 6098-6100

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INTRODUCTION

Pancreatic heterotopia was first described in 1727 when it was found in an ileal diverticulum[1]. It is a rare entity, defined as the presence of extrahepatic tissue without any anatomic or vascular continuity with the pancreas. It may occur at a variety of sites in the gastrointestinal tract having a propensity to affect the stomach and small intestine. Usually, it is a silent anomaly but it may become clinically evident when complicated by inflammation, bleeding, obstruction or malignant transformation[2]. We report a case of a 40-year-old female with an ectopic pancreatic lesion in the antrum of the stomach.

CASE REPORT

A 40-year-old woman was admitted to our hospital due to a 2-mo history of recurrent episodes of epigastric pain, nausea and vomiting. Physical examination, routine blood tests including amylase, plain chest and abdominal X rays along with abdominal ultrasound were unremarkable. Esophagogastroduodenoscopy revealed a sessile polypoid mass with benign features located in the gastric antrum to the posterior wall measuring approximately 2 cm in diameter. The mucosa appeared normal throughout the stomach. Biopsy confirmed the presence of a normal gastric mucosa over the lesion. Computed tomography was not performed.

A decision was made to proceed with surgery. Endoscopic injection with methylene blue was performed to mark the lesion preoperatively.

The patient underwent exploratory laparotomy. Through a small midline incision a gastrotomy was performed. The lesion was clearly stained with methylene blue 4 h after the endoscopy. It was located approximately 10 cm from the pylorus to the greater curvature. In palpation the lesion was rubber like, fixed to the surrounding mucosa giving the feeling 'like a breast fibroadenoma'. Its dimension was approximately 5 cm × 3 cm × 4 cm. By using a stapler device a wedge resection of the lesion was performed with macroscopically clear margins. Frozen sections excluded malignancy and the possibility of ectopic pancreatic lesion. The surgical margins were clear.

The patient had no postoperative complications and was discharged 4 d later. She has remained free of symptoms with negative endoscopy since then.

Histopathologic examination of the lesion showed heterotopic pancreatic tissue in the gastric antrum with a lobular architecture characteristic of ectopic pancreas. The pancreatic lobules were located mainly in the gastric submucosa (Figure 1A) and partially in the muscularis propia (Figure 1B). They contained a mixture of pancreatic acini, ducts and islets of Langerhans. The overlying gastric mucosa was normal.

DISCUSSION

Ectopic pancreas is defined as pancreatic tissue that lacks anatomical or vascular communication with the normal

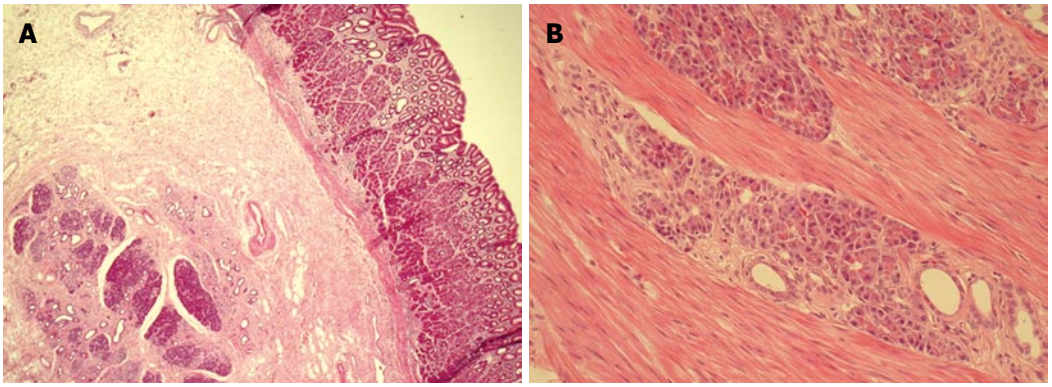

Figure 1 Heterotopic pancreatic lobules occupying the submucosa under an intact normal gastric mucosa (HE, x 100) (**A**) and between smooth muscle fibers of the gastric muscularis propria (HE, x 200) (**B**).

body of the pancreas[3]. Heterotopic pancreas has a genetic make-up, physiologic function, and local environmental exposure similar to that of the pancreas[4]. The incidence in autopsies ranges 0.5%-13.7%, being more common at the age of 30-50 years with a male predominance[3].

Of the 105 gastrectomies performed in our institution over the last five years, ectopic pancreatic tissue was found in only one case (1/105, 0.9%).

Several theories have been proposed to explain the pathogenesis and occurrence of pancreatic heterotopia. The most tenable theory implicates that during the development of normal pancreas from several evaginations, originating from the wall of the primitive duodenum, one or more evaginations may remain in the bowel wall. Migration of this embryonic remnant along with the development of the gastrointestinal tract gives rise to the ectopic pancreatic tissue[5]. Another theory suggests that during embryogenesis pancreatic metaplasia of the endodermal tissues localized in the gastric submucosa may occur[5].

Histopathologically, it is not a diagnostic problem when pancreatic acini, ducts, islets of Langerhans and intervening connective tissue are present. The most characteristic gross feature is a central ductal orifice[6].

Specifically in the stomach, the involvement of submucosal layer, muscularis and subserosal layer is 73%, 17% and 10%, respectively[7]. In the presented case the pancreatic tissue involved both the submucosa and muscularis propria. Heinrich in 1909 proposed three types of heterotopic pancreas but his classification was modified by Gaspar-Fuentes in 1973 acquiring its final form. Type Ⅰ heterotopia consists of typical pancreatic tissue with acini, ducts, and islet cells similar to those seen in normal pancreas (Figure 1). Type Ⅱ heterotopia is composed of pancreatic ducts only, referred as canalicular variety. Type Ⅲ heterotopia is characterized by acinar tissue only (exocrine pancreas). Type Ⅳ heterotopia is made up of islet cells only (endocrine pancreas)[8].

The usual location is in the stomach in 25%-38% of the cases, duodenum in 17%-36%, jejunum in 15%-21.7% and rare in the esophagus, gallbladder, common bile duct, spleen, mesentery, mediastinum and fallopian tubes. Gastric lesions are discovered in the antrum in 85%-95%, either on the posterior or anterior wall, being more common along the greater curvature[5].

The pancreatic ectopic tissue is usually silent but can also undergo complications that occur in normal pancreatic tissue such as acute or chronic pancreatitis, abscess and pseudocyst formation[9]. Malignant transformation may rarely occur. Up to 15 cases have been reported so far[10]. In order to be described as arising from heterotopic pancreas, the diagnosis of a carcinoma should fulfil three criteria: (1) the tumour must be located within or very close to the ectopic pancreatic tissue, (2) transition between pancreatic structures and carcinoma must be identified and (3) the non-neoplastic pancreatic tissue must comprise fully developed acini and ducts[11]. Adenocarcinomas arising from ectopic pancreas seem to have a somewhat better prognosis than those arising from the pancreas itself, probably due to earlier presentation[10].

Symptoms depending upon the anatomical location, such as gastric outlet obstruction in a pre-pyloric rest or obstructive jaundice in a bile duct focus, may originate from the mass effect of the tumour[12] and are also related to the size of the lesion. Lesions greater than 1.5 cm in diameter are more likely to cause symptoms[12]. Pain is one of the most common symptoms. The possible explanation is that the pain is due to endrocrine and exocrine function of the heterotopic pancreatic tissue, and relates to the secretion of hormones and enzymes, being responsible for inflammation or chemical irritation of the involved tissues[13]. Haemorrhage due to mucosal erosion, ulcer formation and perforation especially localized in the small intestine have also been reported[12].

Barium swallow study may show a typical image of a rounded filling defect with central indentation. The reported sensitivity and specificity are 87.5% and 71.4%, respectively[14]. Upper GI endoscopy can demonstrate a broad based umbilicated submucosal lesion. In the majority of cases, biopsies are superficial and non diagnostic. However, positive biopsies can establish the diagnosis[15]. Endoscopic ultrasonography has proven to be a useful adjunct in identification of pancreatic rests, localizing in the submucosa and ranging 0.5-2 cm. The combination of endoscopic ultrasonography with fine-needle aspiration allows cytologic evaluation of submucosal gastrointestinal lesions, having a sensitivity ranging 80%-100%[16,17].

Computed tomography findings are usually non specific. However, multi-slice spiral CT with oral and portovenous phase Ⅳ contrast may demonstrate the lesion which enhances similarly with the normal pancreatic tissue. CT can localize lesions with normal pancreatic tissue but cannot distinguish ectopic pancreas from other submucosal tumors[18,19].

In our case, since neither CT nor endoscopic ultra-

sonography was performed and the biopsy showed normal gastric mucosa, the diagnosis was made based on the benign endoscopic features of the lesion.

The diagnosis may be sometimes difficult intraoperatively due to the gross similarity of pancreatic heterotopia with gastrointestinal stromal tumour (GIST), gastrointestinal autonomic nerve tumour (GANT), carcinoid, lymphoma or even gastric carcinoma. If in doubt, frozen section is very helpful to establish the diagnosis intraoperatively and to avoid unnecessary extensive operations.

In conclusion, although pancreatic heterotopia is rare, it should be always considered in the differential diagnosis of extramucosal gastric lesions. Despite the development of modern diagnostic modalities, its diagnosis remains challenging. Surgical excision provides symptomatic relief and is recommended especially if diagnostic uncertainty remains. If in doubt, frozen section can help to avoid unnecessary radical operations.

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CASE REPORT

Large solitary retroperitoneal echinococcal cyst: A rare case report

Konstantinos Tepetes, Gregory Christodoulidis, Michael Spryridakis, Konstantinos Hatzitheofilou

Konstantinos Tepetes, Gregory Christodoulidis, Michael Spryridakis, Konstantinos Hatzitheofilou, General Surgery Department, University Hospital of Larissa, Mezourlo, Larissa, Greece
Correspondence to: Konstantinos Tepetes, General Surgery Department, University Hospital of Larissa, Mezourlo, Larissa, Athinas 2 Str. Larissa, Greece. gregchrmd@in.gr
Telephone: +30-2410-682729
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Abstract

Echinococcal disease remains a problem within some endemic areas. Echinococcal cysts usually involve the liver and lungs, but any other organ can potentially be involved. Extrahepatic localization is reported in 14%-19% of all cases of abdominal hydatid disease. We report the case of a large echinococcal cyst localized in the lower pelvis. A 28-year-old woman was admitted to a surgical ward with lower abdominal pain and discomfort lasting for a month. Ultrasonography and computed tomography scanning revealed a large retroperitoneal cystic mass (9 cm × 4 cm) in contact with the left ovary and left ureter. There were no cysts in any other location. Serological tests were positive for Echinococcus. The patient was operated on and the entire cyst was excised intact. Histopathological results confirmed the diagnosis of echinococcosis. Antihelminthics were administered postoperatively and the patient was discharged after 6 d, and is now being closely followed up. Total cystectomy when possible represents the treatment of choice for large extrahepatic echinococcal cysts.

Key words: Echinococcus cysts; Ultrasonography; Extrahepatic location; Seropositivity; Antihelminthics; Total cystectomy

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INTRODUCTION

Echinococcal disease, which produces unilocular cystic lesions, is an infection of humans caused by the larval stage of *Echinoccocus granulosus*. It is prevalent in the Middle East, the Mediterranean region, particularly in Greece and Lebanon, Australia, Argentina and Africa. Confirmed hosts are dogs that pass eggs into their feces. Intermediate hosts, e.g. sheep, cattle, humans, goats and horses, ingest the eggs and develop cysts[1]. Echinococcal cysts are mostly found in the liver (60%-70% of cases), followed by the lungs (10%-25%), spleen, ovaries, kidneys, brain, bones and heart, but rarely elsewhere in the body[1]. Hydatid disease in extrahepatic locations usually remains asymptomatic unless the cyst grows and produces symptoms due to pressure, rupture to the pleural or peritoneal cavity, secondary infection, or an allergic reaction[2]. We report the rare case of a 28-year-old woman with a large hydatid cyst in her lower pelvis, without hepatic or any other involvement.

CASE REPORT

A 28-year-old woman was admitted to our surgical ward with vague abdominal pain localized in the hypogastrium, and severe constipation, which had lasted for the previous 20 d. Her gynecologist had diagnosed a large cystic mass adjacent to the left ovary. She did not report episodes of fever, vomiting or diarrhea. Neither did she note any alterations in her menstrual cycle. Blood tests showed mild leukocytosis (12 000 white blood cells). Plain abdominal X-rays did not show any specific diagnostic findings. Abdominal ultrasonography (US) revealed a large cystic mass (9 cm × 4 cm) in contact with the left ovary and left ureter. Computed tomography (CT) confirmed the US findings (Figure 1). There were no cysts in any other location. Serological tests were positive for echinoccocal disease. The patient underwent a laparotomy, and a large cystic mass was identified retroperitoneally, firmly attached to the sacrum and in contact with the left ovary, left ureter and the sigmoid colon. Total cystectomy was performed en block with a portion of the mesosigmoid. The sigmoid vasculature remained intact, and there was no evidence of intraoperative colon ischemia. Postoperatively, antihelminthics were administered and the patient was discharged after 6 d. She had been closely followed up for 3 mo, without any abdominal pain and with significant improvement of constipation.

DISCUSSION

Echinococcosis remains a problem in endemic areas. *E.*

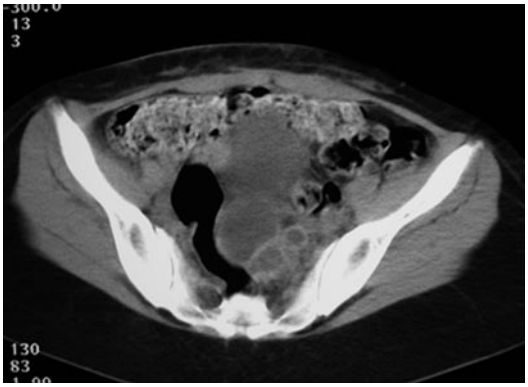

Figure 1 CT image of a large retroperitoneal echinococcal cyst in contact with the left ovary and left ureter.

granulosus is a 5-mm long worm, with a lifespan of 5-20 mo within the jejunum of dogs. When eggs are ingested by an intermediate host, the embryos escape, penetrate the intestinal mucosa, enter the portal circulation, and are then trapped in the liver[3]. A small number escape the hepatic filter, enter the systemic circulation, and are scattered to other organs. Larvae develop into fluid-filled unilocular hydatid cysts that consist of an external membrane and an inner germinal layer. Daughter cysts originate from the inner layer[3,4]. Slowly enlarging echinococcal cysts generally remain asymptomatic. Five to 20 years elapse before cysts enlarge sufficiently to cause symptoms. Abdominal pain, hepatomegaly or a palpable mass in the right upper quadrant are the most common symptoms for patients with liver echinococcosis[1]. Bile-duct compression or leakage of fluid into the biliary tree may mimic cholelithiasis. Biliary obstruction can result in jaundice. Generalized toxic reaction due to hydatid cyst rupture and secondary infection are among the most common complications[5].

Cysts in the peritoneal cavity account for 10%-16% of cases and are mainly the result of the rupture of concomitant hepatic cysts[2]. Extrahepatic locations of the echinococcus include the lungs (10%-15%), spleen (0.9%-8%), kidneys (1%-4%), pancreas (0.25%-0.75%), brain, heart, ovaries, bones and abdominal wall. Symptoms in such cases occur because of pressure or complications including rupture, allergic reaction and secondary infection[6]. Radiography, US and CT studies are important for a diagnosis of echinococcal disease. Plain abdominal X-rays may show calcifications of the cystic wall[7]. US is the method of choice for the detection of hepatic and extrahepatic echinococcal cysts. Hydatid cysts are classified by ultrasound into six categories.

Type I are defined as univesicular and < 50 mm in diameter. Type Ⅱ are univesicular with a prominent laminated layer, and tend to be seropositive. Type Ⅲ are subdivided into Ⅲa, defined as cysts with a prominent lamination that contains daughter cysts, and Ⅲb, characterized by lamination but a lower number of daughter cysts. Both Ⅲa and b are highly seropositive. Type Ⅳ appear as solid masses. Type Ⅴ are characterized by degeneration with calcifications. Type Ⅵ are defined as multiple cysts that may be univesicular and laminated, with daughter cysts involving one or more organs[8]. The sensitivity of US ranges from 93% to 98%[5].

CT confirms the diagnosis by revealing the presence of daughter cysts and plaque-like calcifications in the cystic wall. It is important as it provides information regarding the exact location of extrahepatic cysts in relation to neighboring structures. CT sensitivity ranges from 90 to 97%[5,9].

Serological tests contribute to diagnosis. Immunoglobulin G antibody detection by ELISA has a sensitivity of 95% and a specificity of 94%[8]. The sensitivity of indirect hemagglutination test has been found to be 87.5%[8,10].

Therapy for extrahepatic echinococcal disease is based on considerations regarding the size, location and manifestations of the cysts, and the overall health status of the patient. Asymptomatic small cysts once diagnosed can be treated with antihelminthic drugs, administered for 28 d in one to eight repeating cycles, separated by drug-free intervals of 2-3 wk[11].

For symptomatic or large hydatid peritoneal cysts, surgery, when feasible, is the principal method of treatment. Surgical treatment can be either radical or conservative. Total cystectomy, whenever possible, is the gold standard. For peritoneal cysts firmly attached to intraperitoneal viscera, unroofing and drainage has been proven to be a safe method[11,12]. It is important that the abdominal cavity is isolated with gauzes soaked in 20% hypertonic saline solution to avoid secondary hydatosis and allergic reaction[11].

In our case, to eradicate the disease, total cystectomy was carried out with meticulous dissection of the left ureter, gonadal vessels, hypogastric nerve plexus and the mesosigmoid vasculature. Thus, the sigmoid remained intact, free from compression, and constipation was greatly improved postoperatively.

In conclusion, a diagnosis of extrahepatic echinococcal disease is more accurate today because of the new imaging techniques available. The treatment of choice for small asymptomatic hydatid cysts is conservative by administration of antihelminthic drugs. Large or symptomatic echinococcal cysts need to be treated by total cystectomy, or unroofing and drainage followed by adjuvant antihelminthic therapy.

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CASE REPORT

Mediastinal tuberculous lymphadenitis presenting as an esophageal intramural tumor: A very rare but important cause for dysphagia

APA Pimenta, JR Preto, AMF Gouveia, E Fonseca, MML Pimenta

APA Pimenta, JR Preto, AMF Gouveia, Serviço de Cirurgia Geral, Porto Medical School and Hospital de São João, Porto, Portugal
E Fonseca, Pathology Department, Porto Medical School and Hospital de São João, Porto, Portugal
MML Pimenta, Radiology Department, Porto Medical School and Hospital de São João, Porto, Portugal
APA Pimenta, E Fonseca, Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal
Correspondence to: Dr. Amadeu Pimenta, Professor, Serviço de Cirurgia Geral-Piso 6, Hospital de São João, E.P.E., Hernâni Monteiro, 4202-451 - Porto,
Portugal. amadeupimenta@hotmail.com
Telephone: +351-22-5512308 Fax: +351-22-5512308
Received: August 15, 2007 Revised: September 22, 2007

Abstract

Dysphagia associated with esophageal mechanical obstruction is usually related to malignant esophageal diseases. Benign lesions are rarely a cause for this type of dysphagia, and usually occur either as an intramural tumor or as an extrinsic compression. Mediastinal tuberculous lymphadenitis is rare in adults, and even more rarely causes dysphagia. We report two cases of dysphagia in adult patients caused by mediastinal tuberculous lymphadenitis, presenting radiologically and endoscopically as an esophageal submucosal tumor. Based on the clinical and imaging diagnosis, the patients underwent a right thoracotomy, and excision of the mass attached to and compressing the esophagus. Pathological examination of the specimens showed a chronic granulomatous inflammation with caseous necrosis, which was consistent with tuberculous lymphadenitis.

Key words: Dysphagia; Tuberculous lymphadenitis; Esophageal tumor; Uncommon dysphagia; Esophageal benign lesion

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INTRODUCTION

In the esophagus, squamous cell adenocarcinoma as the most common type of malignant tumors and leiomyoma as the most common type of mesenchymal neoplasms, represent the frequent causes for dysphagia. However, dysphagia associated with esophageal mechanical obstruction can be caused by several conditions, mainly endoluminal/mucosal lesions, intramural tumors or extrinsic compression.

Despite technological improvements in the diagnostic work-up, the definitive diagnosis of some of these entities, particularly differentiation of submucosal parietal lesions from extrinsic lesions compressing the esophagus, may only be established after surgery[1].

Mediastinal diseases such as sarcoidosis[2], lung cancer, lymphoma, distant metastases[3,4] or mediastinal inflammatory lymph nodes[1,5-9] are the conditions that rarely compress the thoracic esophagus and cause dysphagia.

Some causes for dysphagia should always be considered in the differential diagnosis of dysphagia, because some of them if diagnosed and treated in time will completely cure the patient. We herein report two cases of dysphagia caused by mediastinal tuberculous lymphadenitis (MTL), presenting as esophageal intramural tumors.

CASE REPORT

Case 1

A 40-year-old woman who complained of a six-month history of dysphagia for solid food and paroxystic odynophagia, was admitted to our hospital for further evaluation of a submucosal mass-like lesion seen on an upper gastrointestinal (GI) endoscopy taken in another hospital. She had no other digestive symptoms, denied weight loss, fever, cough, sputum and night sweating. Her past medical history was unremarkable, namely exposure to tuberculosis and her family history was also unremarkable. Clinical examination was irrelevant, and her hematological and biochemical tests were normal.

A chest X-ray did not show any parenchymal and pleural change or mediastinal pathology. A barium swallow esophagogram (Figure 1A) disclosed a two-centimeter protruding lesion on the left lateral aspect of the mid-esophagus, distal to the aortic arch and with a smooth surface. An upper GI endoscopy (Figure 1B) showed a

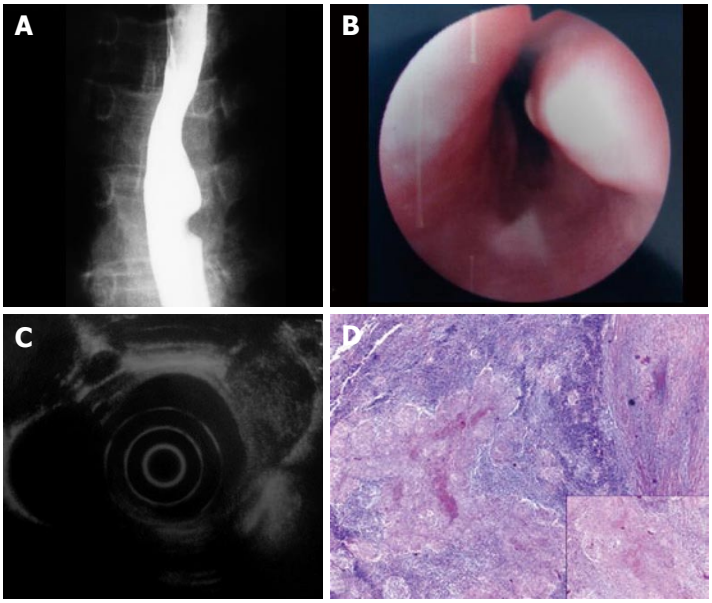

Figure 1 Barium swallow showing a two-centimeter protruding lesion on the left lateral aspect of the mid-esophagus (**A**), and endoscopic view of a two- centimeter protruding lesion covered by normal mucosa and with firm consistency (**B**), endoscopic ultrasonography revealing a two-centimeter solid hypoechoic lesion in the third layer (**C**), and lymph node parenchyma extensively occupied by necrotizing epithelioid granulomas (HE, × 40) (**D**). Insert: typical tuberculous granuloma with central caseous necrosis and multinucleated cells of Langhans type (HE, × 200).

two- centimeter deformity of the esophageal wall, covered by normal mucosa and with firm consistency, located 25 centimeters from the anterior incisor teeth. An endoscopic ultrasonography (Figure 1C) suggested that the lesion described by the other methods was an extramucosal mesenchymal tumor of the esophagus. A CT scan showed no pulmonary lesions or enlargement of mediastinal lymph nodes. The patient underwent a right thoracotomy, and excision of the mass attached to and compressing the esophagus. Surgery was performed with some difficulty due to surrounding fibrosis.

Pathological examination of the specimen showed a conglomerate of lymph nodes surrounded by chronic granulomatous inflammation with caseous necrosis. Zielh-Neelsen stain of the specimen, however, failed to disclose acid-fast bacilli (Figure 1D).

The symptoms of dysphagia and odynophagia disappeared after excision of the mass and post-operative treatment with a three-regimen anti-tuberculous chemotherapy with isoniazid, ethambutol hydrocloride and rifampicin.

A follow-up barium swallow esophagogram revealed a normal esophagus and the patient was in good health 6 years after an uneventful operation and subsequent discharge.

Case 2

A 54-year-old woman was referred to our hospital with a five-month history of dysphagia for solid food and a diagnosis of a submucosal lesion in the middle third of the esophagus. She had no other digestive symptoms, denied weight loss, fever, cough, sputum and night sweating. Her past medical history was unremarkable, namely exposure to tuberculosis and her family history was also unremarkable. Clinical examination was irrelevant, except for a palpable right supra-clavicular lymph-node. In spite of a repeatedly high erythrocyte sedimentation rate (30-73 mm in the first hour), sputum examination to disclose acid-fast bacilli and all the other routine laboratory tests revealed no abnormal findings.

Fine needle cytology of the supra-clavicular lymphadenopathy showed a necrotic lesion, without malignant cells. Zielh-Neelsen stain was negative. A barium swallow esophagogram (Figure 2A and B) disclosed a four-centimeter protruding smooth lesion on the right lateral aspect of the mid-esophagus, with apparent integrity of mucosa. An upper gastrointestinal (GI) endoscopy (Figure 2C) showed a two-centimeter deformity of the esophageal wall covered by normal mucosa and with firm consistency, located 22 centimeters from the anterior incisor teeth. An endoscopic ultrasonography (Figure 2D) revealed a two-centimeter hipoecogenic mass with well-defined limits, which was apparently dependent on the fourth ecographic layer of the esophagus, suggesting a leiomyoma. A chest X-ray and a CT scan did not show any lung parenchymal or pleural change, but the CT scan revealed enlargement of some mediastinum and pulmonary hilum lymph nodes, even though they did not form conglomerates.

Through a right thoracotomy, the mass compressing the esophagus was removed, in fact, an enlarged lymph node, and some other lymph nodes adherent to the esophageal wall were also removed.

Pathological examination of the specimen showed lymph nodes surrounded by a chronic granulomatous inflammation with caseous necrosis. Zielh-Neelsen stain of the specimen disclosed acid-fast bacilli (Figure 2E).

The patient began a postoperative three-regimen anti-tuberculous treatment with isoniazid, ethambutol hydrocloride and rifampicin.

The symptoms of dysphagia disappeared after treatment and a follow-up barium swallow esophagogram (Figure 2F) revealed a normal esophagus. The patient was in good health 4 years after discharge.

DISCUSSION

The ethiology of dysphagia associated with esophageal mechanical obstruction is usually referred to as

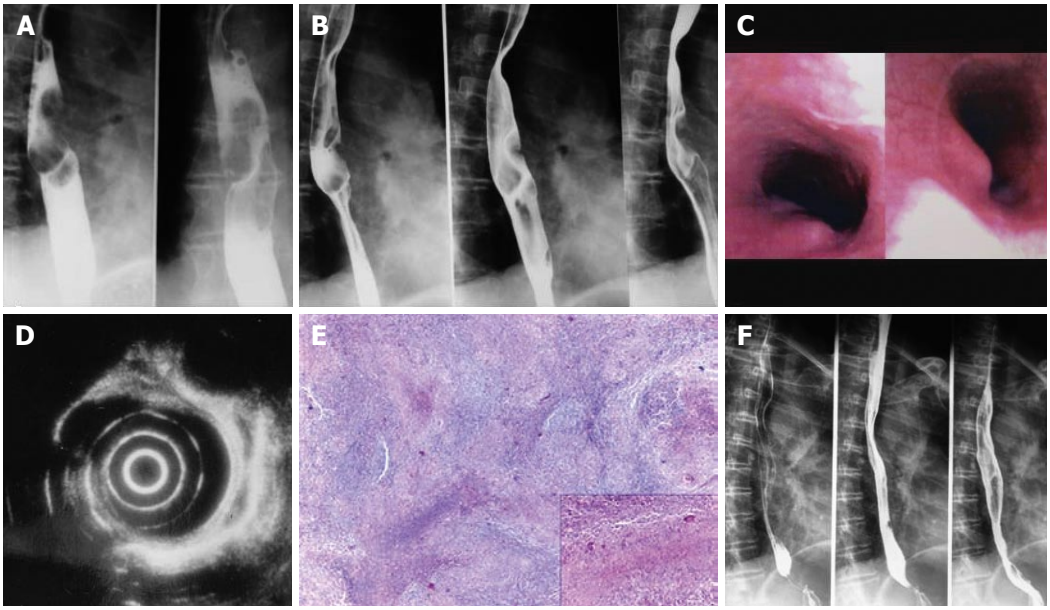

Figure 2 A barium swallow esophagogram disclosing a four-centimeter protruding smooth lesion with apparent integrity of mucosa on the mid-esophagus (**A**, **B**), esophagoscopy showing a two-centimeter deformity of the esophageal wall covered by normal mucosa and with firm consistency, twenty-two centimeters from dental arch (**C**), endoscopic ultrasonography revealing a two-centimeter well defined solid tumor in the fourth layer (**D**), lymph node parenchyma extensively occupied by necrotizing epithelioid granulomas (HE, × 40) and typical tuberculous granuloma with central caseous necrosis and multinucleated cells of Langhans type (HE, × 200) (**E**), and a two-month follow-up after surgery revealing no filling defect at the same level (**F**).

endoluminal/mucosal, intramural or extrinsic, in accordance with the location of the initial lesion in the esophageal wall. Malignant tumors of the esophagus are the most frequent cause, not only for primary dysphagia, but also for dysphagia caused by lesions originating from the esophageal mucosa[10].

Obstructive dysphagia caused by benign lesions is rare and usually results from intramural or extrinsic lesions. Even though rare, benign lesions leading to dysphagia are of great concern in the clinic. In fact, these lesions, if not treated, can lead to death due to progressive obstruction of the esophagus or pulmonary complications, but they almost always allow a curative surgical resection[10].

The endoscopic and radiological characteristics of a submucosa tumor are the endoluminal protusion of the digestive tube wall, usually covered by a normal looking mucosa. However, such features may also be caused by organ lesions or structures that extrinsically compress the digestive wall. EUS is described as the diagnostic method with a greater capacity of distinguishing between intraparietal and extrinsic lesions compressing the digestive wall, with a diagnostic accuracy superior to CT-scan and barium swallow[11,12]. Although EUS-guided fine needle biopsy is possible in these lesions, the material retrieved is sometimes insufficient for a diagnosis and, particularly for evaluation of the malignant potential of the lesions[12-15].

In our cases, even though the diagnosis of an extrinsic compression on the esophagus could not be excluded, barium swallow revealed an image compatible with a submucosa tumor protruding into the lumen. In fact, the angle between the esophageal wall and margin of the mass was almost perpendicular, which is in accordance with an intramural lesion, rather than an extrinsic compression on the esophagus[16]. Furthermore, all of the other diagnostic examinations pointed towards the diagnosis of a submucosal tumor-like intraluminal protruding mass. For these reasons, the patients were operated on with a probable diagnosis of a leiomyoma of the esophagus, suggesting that this is the most frequent intraparietal benign tumor of the esophagus.

One of our patients had a supraclavicular adenopathy and a persistently high erythrocyte sedimentation rate. The lymph node biopsy revealed necrotic material, but no Zielh-Neelson stained bacilli. Even though central necrosis of a lymph node is a frequent finding in tuberculous lymphadenitis[17], this fact does not give us a diagnosis of tuberculosis. In our cases, pulmonary Rx and CT scan of the thorax did not reveal lesions compatible with the diagnosis of pulmonary tuberculosis.

Mediastinal inflammatory lymph nodes, particularly tuberculous lymphadenitis[1,6-9] is a condition compressing the thoracic esophagus and causing dysphagia. Although mediastinal tuberculous lymphadenitis is rare, it is increasing in adults[18-20]. On the other hand, tuberculosis is an infectious disease with a rising incidence particularly in Asian and Eastern European countries and also a rising prevalence in association with HIV infection[21]. In 1993, tuberculosis was declared as a global emergency, by the World Health Organization[22].

Mediastinal tuberculous lymphadenitis should be included in the differential diagnosis of dysphagia, but it should be realized that it can present with various endoscopic and radiological findings. In fact, mediastinal tuberculous lymphadenitis can affect the esophagus by compressing the esophagus externally, causing rupture in the mediastinum and leading to an inflammatory process with secondary involvement of the esophagus, invading

the esophagus, ulcerating the mucosa and draining caseum into the esophageal lumen[9], as well as resulting in an esophageal fistula in some cases[23].

Dysphagia in mediastinal tuberculous lymphadenitis is due to the external compression on the esophagus, but the pain during swallowing - odynophagia occurring in one of our patients, suggested that the esophagus is directly involved in the inflammatory process. Interestingly, Ghmire and Walker[7] reported a case of mediastinal tuberculous lymphadenopathy who had painful dysphagia without significant involvement of the esophagus endoscopically and radiologically. They assumed that the contiguous inflammation of paraesophageal tissues resulted in disturbed esophageal motility of the patient.

In cases of tuberculous adenitis with esophageal mucosal lesion, confirmation of diagnosis should be done by histological or microbiological examination of the specimen obtained by endoscopic biopsy. In our cases without lesion of the mucosa, but with a strong suspicion that the cause for dysphagia is of tuberculous origin, EUS and endoscopic fine needle biopsy can play a role in diagnosing mediastinal masses that produce esophageal symptoms[5]. Other authors prefer biopsies guided by mediastinoscopy or thoracoscopy, when there is no mucosal lesion[24]. Nevertheless, when it is impossible to reach the affected lymph nodes through these approaches, surgery may be necessary in order to establish a diagnosis[1].

At present, minimally invasive surgery should be attempted to remove lesions diagnosed pre-operatively as probable leiomyomas. However, it was difficult, in the first case, to localize the lesion immediately below the aortic arch, on the left side of the esophagus associated with the surrounding fibrosis. In fact, it is hard to isolate the lesion in the presence of mediastinitis fibrosis resulting from rupture of an affected lymph node[25-27]. Medical treatment of most patients with tuberculosis consists of a short course in chemotherapy, using 3 or 4 essential anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, pyrazinamide and streptomycin). Anti TB regimen consists of an initial intensive phase and a continuation phase[22]. It was reported that most patients can be successfully treated with a three-drug anti-tuberculosis chemotherapy regimen[6,8,9,28]. If the diagnosis of mediastinal tuberculous lymphadenitis is made pre-operatively, such a treatment may lead to favourable outcomes[16,24]. Surgery should be reserved for patients with no pre-operative diagnosis, as in our cases, and those who develop complications, such as mediastinal abscess that is unresponsive to non operative management[1].

Since the diagnosis of a small esophageal submucosal lesion or an intraparietal lesion is usually difficult, surgery is necessary. Even though rare, the diagnosis of mediastinal tuberculous lymphadenitis should be highly considered in the presence of an uncertain esophageal lesion[29]. This is particularly important because a diagnostic non surgical approach is possible and an adequate treatment can completely cure mediastinal tuberculous lymphadenitis with or without tuberculous esophagitis[16].

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CASE REPORT

Malakoplakia of the colon associated with colonic adenocarcinoma diagnosed in colonic biopsies

F Karasavvidou, SP Potamianos, S Barbanis, E Stathakis, A Psychos, AN Kapsoritakis, G Koukoulis

F Karasavvidou, S Barbanis, E Stathakis, G Koukoulis, Department of Pathology, University Hospital, Larissa 41110, Greece
SP Potamianos, A Psychos, AN Kapsoritakis, Department of Gastroenterology, University Hospital, Larissa 41110, Greece
Correspondence to: Foteini Karasavvidou, Department of Pathology, University Hospital of Larissa, Larissa 41110, Greece. fotkaras@yahoo.gr
Telephone: +30-2410-681794 Fax: +30-2410-670113
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Abstract

Malakoplakia, typically involving the urinary tract, is an uncommon form of chronic inflammation caused by chronic infections and characterized by accumulation of macrophages. It has also been found in many other sites such as the gastrointestinal tract, pancreas, liver, lymph nodes, skin, respiratory tract, adrenal gland, vagina and brain. We present a case of a 64-year-old man referred to our hospital with cachexia and radiologic evidence of metastatic tumor of the liver. Colonoscopy revealed a large malignant - appearing polypoid mass of the ascending colon and multiple distinct polyps throughout the rest of the colon. Biopsies of the ascending colon mass confirmed the diagnosis of adenocarcinoma. Histological examination of two of the other polyps revealed malakoplakia which was characterized by aggregates of granular histiocytes with Michaelis - Gutmann bodies and histochemically confirmed with periodic acid-Schiff and von Kossa stains. This is a rare case diagnosed on endoscopic samples. The majority of reported cases were found in surgical specimens. In addition, the endoscopic appearance of multiple polyps is unusual in malakoplakia.

Key words: Malakoplakia; Gastrointestinal tract; Colon cancer

Karasavvidou F, Potamianos SP, Barbanis S, Stathakis E, Psychos A, Kapsoritakis AN, Koukoulis G. Malakoplakia of the colon associated with colonic adenocarcinoma diagnosed in colonic biopsies. *World J Gastroenterol* 2007; 13(45): 6109-6111

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INTRODUCTION

Malakoplakia, typically involving the urinary tract, is an uncommon form of chronic inflammation caused by chronic infections and characterized by accumulation of distinct macrophages[1,2]. It has also been found in various sites such as the gastrointestinal tract, pancreas, liver, lymph nodes, skin, respiratory tract, adrenal gland, vagina and brain[3]. We describe a case of a 64-year-old man with end-stage carcinomatosis which originated from the ascending colon and was associated with colonic malakoplakia. The endoscopic appearance of malakoplakia was very unusual, mimicking multiple polyps throughout the large bowel.

CASE REPORT

A 64-year-old man was admitted to our hospital with cachexia, ascites and multiple liver metastases, according to an abdominal CT scan. He reported a three-month history of fatigue, shortness of breath, altered bowel habits, weight loss and progressive abdominal distension. On examination he was pale and cachectic, and had a bulging abdomen with flank and shifting dullness. Blood pressure was 100/65 mmHg and pulse rate 94 beats/min. Hemoglobin was 8.1 g/dL and hematocrit 25%. Blood chemistry revealed cholestasis and 2.6 g/dL albumin and normal CEA levels. Abdominal CT scan revealed widespread hepatic metastases and a large quantity of ascetic fluid. Abdominal paracentesis and ascetic fluid cytology were compatible with peritoneal carcinomatosis. Colonoscopy revealed a large malignant polypoid lesion, almost obstructing the lumen of the ascending colon, as well as multiple (13) distinct polyps throughout the rest of the colon (5 rectal, 5 sigmoid, 3 transverse). The polyps were sessile, soft, ulcerated and hemorrhagic, measuring 5-15 mm in diameter (Figure 1). Two of them were removed in order to exclude a familial polyposis syndrome. The patient received only supportive treatment and subsequently died of liver failure-generalized carcinomatosis two weeks after hospitalization. Biopsies of the ascending colon mass confirmed the diagnosis of adenocarcinoma.

Histological examination of two of the other polyps revealed malakoplakia characterized by aggregates of granular histiocytes (Figure 2A). Several of these histiocytes contained intracytoplasmic Michaelis - Gutmann bodies (Figure 2B), which were confirmed

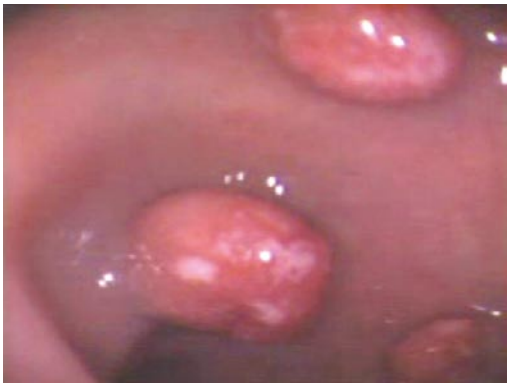

Figure 1 Endoscopic appearance of distinct polyps of the colon.

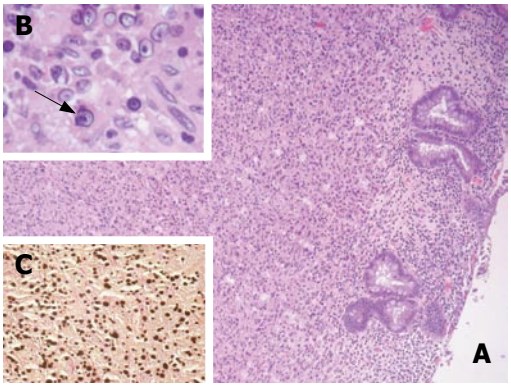

Figure 2 Aggregates of granular histiocytes in the lamina propria of large bowel mucosa (HE, × 200) (**A**), Michaelis - Gutmann bodies (HE, × 400) (**B**) and (Von Kossa, × 200) (**C**).

histochemically with periodic acid-Schiff and von Kossa stains (Figure 2C).

DISCUSSION

Malakoplakia, derived from the Greek adjective malakos (soft) and plaka (plaque), was first described in 1902 by Michaelis and Gutmann[4]. It occurs predominantly in the genitourinary tract (about 75% of the reported cases)[2]. The second most common site is the gastrointestinal tract (11% of the cases), and the majority of these cases involve the rectum and colon[5,6]. The remaining cases affect the brain, lungs, lymph nodes, adrenals, tonsils, conjunctiva, skin, bone, abdominal wall, liver, pancreas and retroperitoneum[3]. An increasing number of cases have been correlated with immunosuppression[7,8].

Definitive diagnosis of the lesion can be made by histopathologic examination. Malakoplakia is characterized by aggregates of histiocytes with abundant eosinophilic cytoplasm known as von Hansemann cells, intermingled with lymphocytes, plasma cells and neutrophiles. Finding the well-known Michaelis-Gutmann bodies is diagnostic for malakoplakia. These bodies are phagolysomes that have become encrusted with calcium and iron salts. They vary in size from 2 μm to 10 μm, have targetoid appearance due to concentric laminations and are stained with periodic acid-Schiff and von Kossa calcium stains. At the ultrastructural level, disintegrated bacteria have been occasionally observed in the Michaelis-Gutmann bodies. The origin of Michaelis - Gutmann bodies is most likely an abnormal response resulting in incompletely digested bacterial fragments and subsequent mineralization[1,9].

Indeed, malakoplakia is related to chronic bacterial infections, such as E*scherichia coli, Proteus mirabilis, Staphylococcus aureus, Mycobacterium Tuberculosis* and *Shigella boydii*. Fungi such as *Paracoccidiodes brasiliensis* and viruses have also been implicated[1,9,10]. In patients with AIDS, *Rhodococcus equi* has also been reported[11]. The pathogenesis of malakoplakia remains unknown. Three possible pathogenetic mechanisms have been suggested: an unusual causative organism, an abnormal or altered immune response and an abnormal macrophage response due to defective lysosomal function[12].

Colonic malakoplakia was first described by Terner and Lattes in 1965[13] and has been reported to occur in conjunction with tumors and non-tumoral conditions[14-16]. Since 1965 about 95 cases of colonic malakoplakia have been published. Notably, 24 of them had a coexistent colonic adenocarcinoma, similarly to our case[6]. All of the reported cases were found in surgical specimens, most of them in conjunction with the tumor. Half of the cases occured as a pericolic mass and only 3 cases as a single nodule or a microscopic focus[15].

However, our patient is a rare case in which the diagnosis of malakoplakia was made preoperatively on biopsy samples as previously described[17,18]. The additional biopsies were prompted by the presence of multiple small polyps in addition to the main cancerous one. The coexistence of multiple colonic polyps related to malakoplakia has not been described previously, the previously reported endoscopic appearances have been described as unifocal or nodular lesions and large masses, and the presence of a pericolonic mass associated with a fistula has been noted[6].

Notably, von Hasselman histiocytes can mimic adenocarcinoma cells in frozen sections. A helpful clue for the correct diagnosis is the presence of Michaelis-Gutmann bodies which are not seen in mucin vacuoles.

Our case may serve as a reminder of the clinical significance of malakoplakia coexisting with colonic adenocarcinoma, which increases the risk of over-staging the tumor and over-treating the patient.

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CASE REPORT

Iatrogenic colorectal perforation induced by anorectal manometry: Report of two cases after restorative proctectomy for distal rectal cancer

Jun-Seok Park, Sung-Bum Kang, Duck-Woo Kim, Na-Young Kim, Kyoung-Ho Lee, Young-Hoon Kim

Jun-Seok Park, Department of Surgery, Chung-Ang University Hospital, Seoul National University Bundang Hospital, Seongnam-si, Gyeonggi-do 463-707, Korea
Sung-Bum Kang, Duck-Woo Kim, Department of Surgery, Seoul National University Bundang Hospital, Seongnam-si, Gyeonggi-do 463-707, Korea
Na-Young Kim, Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam-si, Gyeonggi-do 463-707, Korea
Kyoung-Ho Lee, Young-Hoon Kim, Department of Diagnostic Radiology, Seoul National University Bundang Hospital
Correspondence to: Sung-Bum Kang, MD, Professor, Department of Surgery, Seoul National University Bundang Hospital, 300 Gumi-dong Bundang-gu, Seongnam-si, Gyeonggi-do 463-707, Korea. kangsb@snubh.org
Telephone: +82-31-7877093 Fax: +82-31-7874055
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Abstract

There are no reports regarding perforation of the colorectum induced by anorectal manometry. We report two cases of colorectal perforation that occurred during manometry in the patients undergoing restorative proctectomy for distal rectal cancer. In the first patient, computed tomography showed an extraperitoneal perforation in the pelvic cavity and a rupture of the rectal wall. A localized perforation into the retroperitoneum was managed conservatively. In the second patient, a 3 cm linear colon rupture was detected above the anastomotic site. A primary closure of the perforated colon and proximal ileostomy were conducted, but the patient died 2 wk later. We hypothesize that the perforation induced by anorectal manometry may be associated with the relative weakening of the proximal bowel wall due to anastomosis, decreased compliance, and abnormal rectal sensation. We suggest that measurement of the maximum tolerable volume should not be routinely performed after restorative proctectomy for distal rectal cancer.

Key words: Iatrogenic perforation; Anorectal manometry; Rectal cancer; Low anterior resection

Park JS, Kang SB, Kim DW, Kim NY, Lee KH, Kim YH. Iatrogenic colorectal perforation induced by anorectal manometry: Report of two cases after restorative proctectomy for distal rectal cancer. *World J Gastroenterol* 2007; 13(45): 6112-6114

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INTRODUCTION

Anorectal manometry is an objective test for evaluating a patient's resistance to spontaneous defecation provided by the sphincter mechanism, as well as the sensory capabilities of the rectum in terms of the sensation of imminent defecation[1]. Currently, anorectal manometry is widely performed for the tracking of anorectal physiological changes occurring after low anterior resection for rectal cancer, as the test allows for a numerical value evaluation of pre- and post-operative anorectal function, including rectoanal inhibitory reflex, rectal compliance, and anal resting pressure[2-4]. This procedure is generally thought to be safe, and the incidence of critical complications associated with anorectal manometry has not been reported. We recently encountered two unusual cases of iatrogenic perforation occurring following anorectal manometry in rectal cancer resection patients.

CASE REPORT

Case 1

A 72-year-old male patient received ultra-low anterior resection with coloanal anastomosis for the treatment of rectal cancer 22 mo ago. The patient's primary tumor was located 4 cm from the anal verge. He complained of frequent defecation in excess of 10 bowel movements a day, as well as urgency and tenesmus. We performed anorectal manometry in order to measure changes in the patient's anorectal function. Anorectal manometry (Model UPS-2020 Stationary GI Motility System, MMS, Netherlands) was conducted using the water-perfusion technique, with an 8-channel micro tip catheter connected to a perfusion pump. We evaluated the rectal sensation *via* inflation of a latex balloon with an air flow of 1 mL per second. The threshold volumes for the first minimum sensation, defecatory desire, urge, and maximum tolerance were determined. In this study, the maximum resting pressure (48.25 mmHg) determined was significantly lower than that observed in the normal controls (normal value:

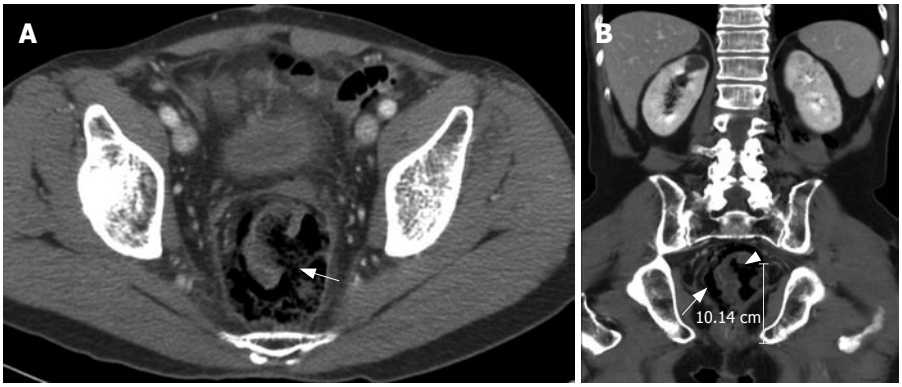

Figure 1 Abdomino-pelvic CT at the level of acetabula showing air bubbles mixed with small solid particles surrounding both lateral aspects of the rectum (arrow) (**A**) and coronal view of extraluminal air in the pelvic cavity (arrow) and rupture of the left lateral wall of the rectum (arrow head) (**B**).

53-90 mmHg), and the maximum squeezing pressure (117 mmHg) was not reduced in comparison with the normal controls (normal value: 100-200 mmHg). During the test, the patient complained of slight discomfort in the lower abdomen during measurement of the maximum tolerable volume. When the balloon catheter was removed, however, the surface of the balloon observed was slightly blood stained. As the patient had normal vital signs and appeared to be relatively healthy, he was discharged after examination. Seven hours later, the patient revisited the emergency room because of persistent lower abdominal pain, anal pain, and a sensation of "chilling". Upon physical examination, the patient experienced mild lower abdominal tenderness with palpation but no symptoms of generalized peritonitis. His temperature was 39.2℃ initially, and decreased within three hours to 38.5℃. His heart rate was 110 per minute and no hypotension was found. The most noteworthy feature of his laboratory studies was an elevated white blood cell count of 17 000/mm^3. Upright chest and abdomen films were normal. However, abdominal CT showed a moderate amount of extraperitoneal air in the pelvic cavity and a rupture of the rectal wall (Figure 1A and B). Perforation into the retroperitoneum was localized, and no signs of intraperitoneal perforation were observed. The patient was hospitalized and received no treatment by mouth, total parenteral nutrition, and intravenous broad-spectrum antibiotics. Daily physical examinations were conducted. We verified improvement in radiologic signs on a CT examination conducted seven days later. The patient was discharged on the 14th d of hospitalization.

Case 2

A 78-year-old female patient underwent an ultra-low anterior resection and coloanal anastomosis following preoperative radiotherapy (50.4 Gy during 5 wk) coupled with infusion of 5-FU for low rectal cancer 23 mo ago. The patient had a history of angioplasty due to unstable angina 4 years ago. She presented at the hospital for frequent defecation and urgency to defecate, which persisted after surgery. We performed anorectal manometry to measure the function of her anorectum in the same manner as in Case 1. No abnormalities were detected with the exception of loss of rectoanal inhibitory reflex and a reduction in resting pressure. However, when the rectal balloon was gradually inflated with 130 mL air for measurement of the maximum tolerable volume, a steep fall in intra-balloon pressure (from 130 mmHg to 65 mmHg) was detected, and the examiner could actually feel her resistance against the decrease in air injection. During the test, she complained of an abrupt discomfort in the abdomen and abdominal distension. An urgent CT scan of the abdomen and pelvis was conducted, which evidenced a large quantity of free intraperitoneal gas and fluid within the abdomen consistent with the perforation of a gas-containing viscous body (Figure 2). Emergency laparotomy was immediately conducted, and a 3 cm linear colon rupture was detected above the coloanal anastomosis suture area. Accordingly, primary closure of the perforation site and a diverting ileostomy were performed. The patient's underlying heart condition deteriorated rapidly after surgery, and she died two weeks later, despite aggressive resuscitation.

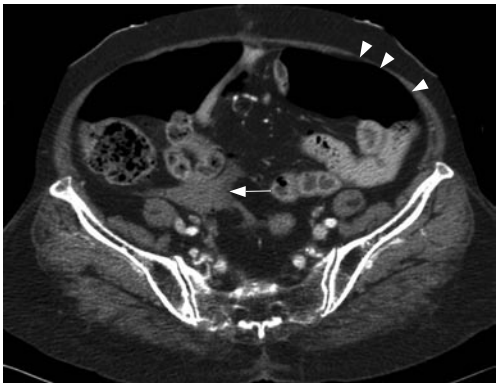

Figure 2 Abdomino-pelvic CT at pelvis level showing a large amount of extraluminal air (arrow heads) mixed with a complicated collection of fluids, probably primary extraluminal feces (arrow).

DISCUSSION

We experienced two iatrogenic colorectal perforations (0.13%) in 1501 anorectal manometry tests in the past three years. Both patients had a history of rectal cancer resection. Anorectal manometry has been widely adopted as a means for evaluating physiological changes in the anus and rectum of patients undergoing low anterior resection. To our knowledge, no iatrogenic perforation has been reported as a complication arising from anorectal manometry conducted following low anterior resection[3,5-7].

We consider that this colorectal perforation is

associated with certain characteristics of the neorectum following low anterior resection and anastomosis, including relative weakening of the proximal bowel wall due to anastomosis, decreased compliance, and abnormal rectal sensation. The pressure of balloon inflation can exert undue stress on the weakened proximal bowel wall to fibrotic anastomosis, causing rupture on the neorectum. The vulnerable part, which evidences low compliance, can be readily ruptured by the application of physical force via artificial balloon inflation. As the rectal balloon is inflated, patients are instructed to inform the examiner of the rectal sensation according to changes in the air injection level. However, patients with dull rectal sensation are not able to appropriately express it. In the case of the aged, who undergo rectal surgery or to whom radiotherapy is administered, there is some risk that the balloon may be inflated over the actual maximum threshold volume.

In treatment of iatrogenic colonic perforation, nonoperative management of colonic perforation is advocated for patients who are clinically stable with no evidence of peritonitis[8-10]. For selected patients with incidental intramural or small retroperitoneal perforations but no evidence of barium spillage, favorable results have also been reported as the result of conservative treatment consisting of bowel rest combined with total parenteral nutrition, intravenous fluid treatment, and broad-spectrum antibiotics[11,12]. On the basis of our experience with the two cases, this indication for conservative management after iatrogenic perforation may also be applied to perforation occurring during anorectal manometry. However, we believe that there may be a higher risk for perforation during anorectal manometry than for other types of perforation because (1) anorectal manometry is conducted without reasonable bowel preparation and (2) diagnostic delays are likely to occur as physicians tend not to recognize the possibility of perforation. Therefore, a more cautious approach should be taken when selecting patients who can receive conservative treatment for perforation occurring during anorectal manometry.

In order to avoid iatrogenic perforations during anorectal manometry, it is important to assess the high risk factors associated with perforation prior to anorectal manometry. History taking should focus on age, previous rectal surgery, bowel inflammation, and bowel obstruction. Meticulous digital rectal examination preceding anorectal manometry, for the detection of unsuspected anorectal abnormal lesions, is necessary for patients with a history of rectal surgery. This facilitates catheter insertion and provides information on anorectal conditions. We believe that the process of measuring the maximum tolerable volume may be omitted in patients following low anterior resection and anastomosis for distal rectal cancer. The maximum tolerable volume may be highly distorted in patients undergoing rectal resection in comparison with patients with normal rectum, as sensations of rectal distension differ in accordance with the patterns and rates of balloon inflation, which are dependent on examiners and laboratories[13]. We suggest that measurement of the maximum tolerable volume should not be routinely performed in patients undergoing restorative proctectomy for distal rectal cancer.

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CASE REPORT

Epidermal growth factor receptor antibody plus recombinant human endostatin in treatment of hepatic metastases after remnant gastric cancer resection

Long Sun, Huang-Yang Ye, Ying-Hong Zhang, Yong-Song Guan, Hua Wu

Long Sun, Hua Wu, Minnan PET Center, The First Hospital of Xiamen, Fujian Medical University, Xiamen 316003, Fujian Province, China
Huang-Yang Ye, Ying-Hong Zhang, Department of Oncology, The First Hospital of Xiamen, Fujian Medical University, Xiamen 316003, Fujian Province, China
Yong-Song Guan, State Key Laboratory of Biotherapy, West China Medical School, Sichuan University, Chengdu 610041, Sichuan Province, China
Correspondence to: Hua Wu, Minnan PET Center, The First Hospital of Xiamen, Fujian Medical University, Xiamen 316003, Fujian Province, China. wuhua1025@163.com
Telephone: +86-592-2139527 Fax: +86-592-2139527
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Abstract

We report a 55-year-old male who developed advanced hepatic metastasis and peritoneal carcinomatosis after resection of remnant gastric cancer resection 3 mo ago. The patient only received epidermal growth factor (EGF) receptor antibody (Cetuximab) plus recombinant human endostatin (Endostar). Anti-tumor activity was assessed by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography/computer tomography (PET/CT) at baseline and then every 4 wk. The case illustrates that ^{18}FDG-PET/CT could make an early prediction of the response to Cetuximab plus Endostar in such clinical situations. ^{18}FDG-PET/CT is a useful molecular imaging modality to evaluate the biological response advanced hepatic metastasis and peritoneal carcinomatosis to Cetuximab plus Endostar in patients after remnant gastric cancer resection.

Key words: Hepatic metastasis; Remnant gastric cancer; Cetuximab; Recombinant human endostatin; ^{18}F-fluorodeoxyglucose; Positron emission tomography/computer tomography

Sun L, Ye HY, Zhang YH, Guan YS, Wu H. Epidermal growth factor receptor antibody plus recombinant human endostatin in treatment of hepatic metastases after remnant gastric cancer resection. *World J Gastroenterol* 2007; 13(45): 6115-6118

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INTRODUCTION

The activity of epidermal growth factor (EGF) and its receptor (EGFR) has been identified as the key driver in the process of cell growth and replication. There is now a body of evidence that EGFR-mediated drive is increased in a wide variety of solid tumors, including non-small cell lung cancer, prostate cancer, breast cancer, gastric cancer, colon cancer, ovarian cancer and tumors of the head and neck[1]. EGFR antibody (Cetuximab, Erbitux, Merck and Imclone Systems) has been approved by the Food and Drug Administration (FDA)[2] for use in treatment of colorectal cancer. Angiogenesis is the formation of new capillaries from existing blood vessels. The recognition of tumor angiogenesis as a therapeutically useful target is based on experimental evidence that tumor growth and progression are dependent on new blood vessel formation. Endostatin has been shown to inhibit potently angiogenesis *in vitro* and *in vivo* and Endostar (Medgenn Bioengineering Co. Ltd. Yantai, Shandong, P. R.China.) has been approved by the State Food and Drug Administration (SFDA)[3]. In this case, we used ^{18}FDG-PET/CT to monitor the early response of advanced hepatic metastasis and peritoneal carcinomatosis to Cetuximab plus Endostar in patients after remnant gastric cancer resection.

CASE REPORT

A 55-year-old male, with a history of subtotal gastrectomy for gastric ulcer 20 years ago, received remnant gastric cancer resection 3 mo ago. Immunohistochemistry revealed adenocarcinoma of the stomach with expression of EGFR. Episodes of fever above 40℃ appeared without evidence of infection and mild jaundice developed with weight loss of 10 kg within 1 mo. A palpable liver and tenderness of the left upper abdominal quadrant were found during physical examination. Laboratory findings during the first admission showed 55 g/L HGB, 41.4 μmol/L TBIL, 12.9 μmol/L DBIL, and 28.5 μmol/L IBIL. Advanced hepatic metastasis and peritoneal carcinomatosis were confirmed by ^{18}F-fluorodeoxyglucose positron emission tomography/computer tomography (^{18}F-FDG PET/CT) and biopsy guided by ^{18}F-FDG PET/CT. These findings were supportive for tumor-associated cachexia because his overall performance status was significantly reduced (Karnofsky index: 40%). Conventional chemotherapy was not considered the first choice of

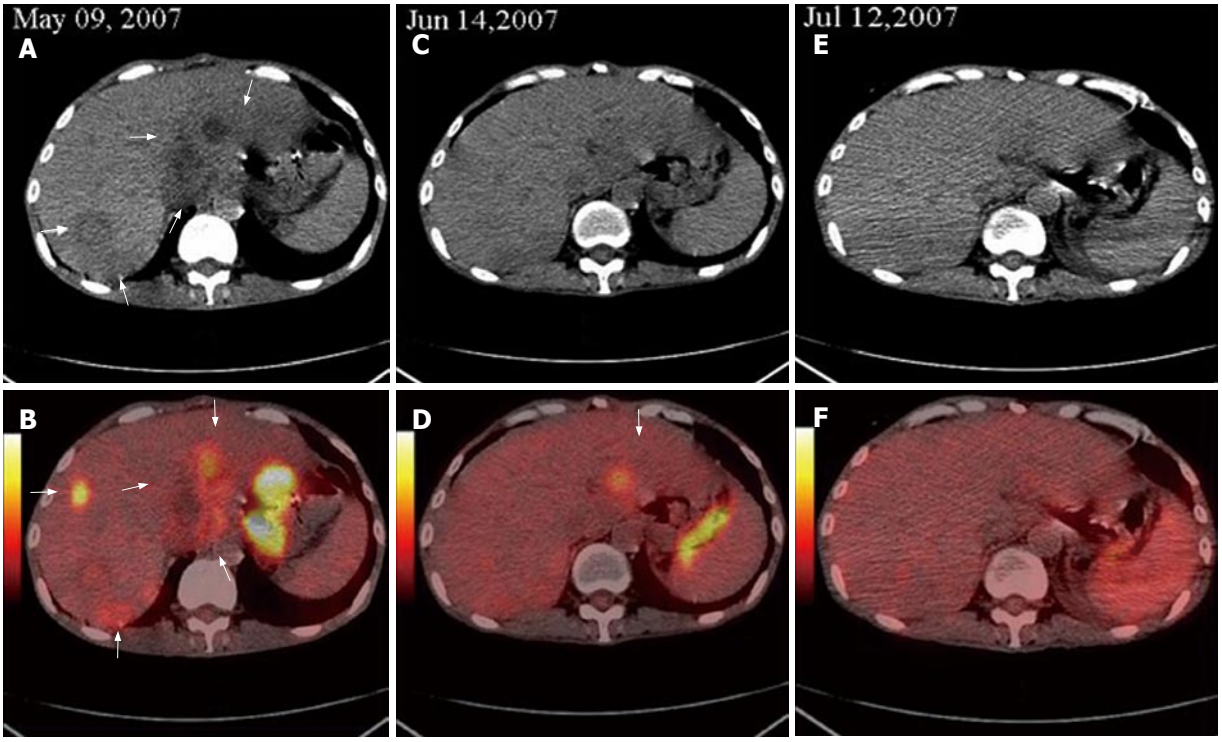

Figure 1 Base line FDG PET/CT detecting a highly metabolic metastasis in liver (white arrows, **A** and **B**), second FDG PET/CT demonstrating the decreased number and metabolism level of liver metastases and one highly metabolic metastasis in the left lobe of liver (white arrows, **C** and **D**), third FDG PET/CT displaying no lesion at the same position (**E** and **F**).

treatment for the patient. After giving informed consent, the patient received Cetuximab weekly at 400 mg/m^2 iv loading dose, followed by at 250 mg/m^2 iv maintenance dose for 8 wk, together with but separately used Endostar daily at 15 mg iv loading dose and maintenance dose for over 8 wk. Anti-tumor activity was assessed by ^{18}F-FDG PET/CT at baseline and then every 4 wk. During the 8-wk follow-up period of time, ^{18}F-FDG PET/CT was performed 3 times to monitor the treatment response. His temperature returned to normal slowly after 4 wk of treatment. Cetuximab exhibited only mild skin toxicity, and hepatic metastasis and peritoneal carcinomatosis lesions had a partial response to Cetuximab treatment (> 30% reduction, according to RECIST) after 4 wk (Figure 1 and Figure 2). Main laboratory tests showed 70 g/L HGB, 21.4 μmol/L TBIL, 13.9 μmol/L DBIL, 16.5 μmol/L IBIL. Karnofsky index was 70%. Due to responses to the treatment without serious complications, after the second treatment cycle, hepatic metastases were almost completely gone on the third ^{18}F-FDG PET/CT (Figures 1 and 2). However, a few metastatic lymphoid nodes in the abdomen could be detected (Figure 3). In parallel, main laboratory tests showed 90 g/L HGB, 20.4 μmol/L TBIL, 12.9 μmol/L DBIL, 15.5 μmol/L IBIL. Karnofsky index was 80%. Due to the confirmed responses to the treatment, the third cycle of treatment was going on when this paper was completed.

DISCUSSION

Multiple cellular pathways involved in the growth and metastatic potential of tumors may create heterogeneity, redundancy, and the potential for tumors to bypass signaling pathway blockade, resulting in primary or acquired resistance. Combined therapies are more effective in inhibiting different signaling pathways and can overcome tumor resistance[4]. Vascular endothelial growth factor (VEGF) and EGFR inhibitors have become the key components of therapies for several tumor types. There is a close relationship between these two factors. VEGF signaling is up-regulated by EGFR expression while VEGF up-regulation independent of EGFR signaling seems to contribute to resistance to EGFR inhibition. Therefore, inhibition of both pathways improves anti-tumor efficacy and overcomes resistance to EGFR inhibition[5].

EGFR belongs to a family of receptors known as the ErbB family (ErbB tyrosine kinase receptors), which comprises four proteins encoded by the c-erbB proto-oncogene. EGFR can activate a cascade of multiple signaling pathways that facilitate tumor growth process[6]. The EGFR signaling pathway regulates cell differentiation, proliferation, migration, angiogenesis, and apoptosis, all of which are down regulated in cancer cells. In a study, EGFR immunoreactivity was detected in one (3.8%) of the 26 early gastric carcinomas and in 33 (34.4%) of the 96 advanced gastric carcinomas, respectively, the incidence of expression between the two groups was significantly different[7]. In gastric cancer, the management of peritoneal dissemination in the peritoneal cavity is extremely important. However, peritoneal dissemination in the final stage of gastric cancer remains untreatable. VEGF is correlated with peritoneal metastasis from gastric cancer, and has been reported as a useful indicator of peritoneal recurrence[8].

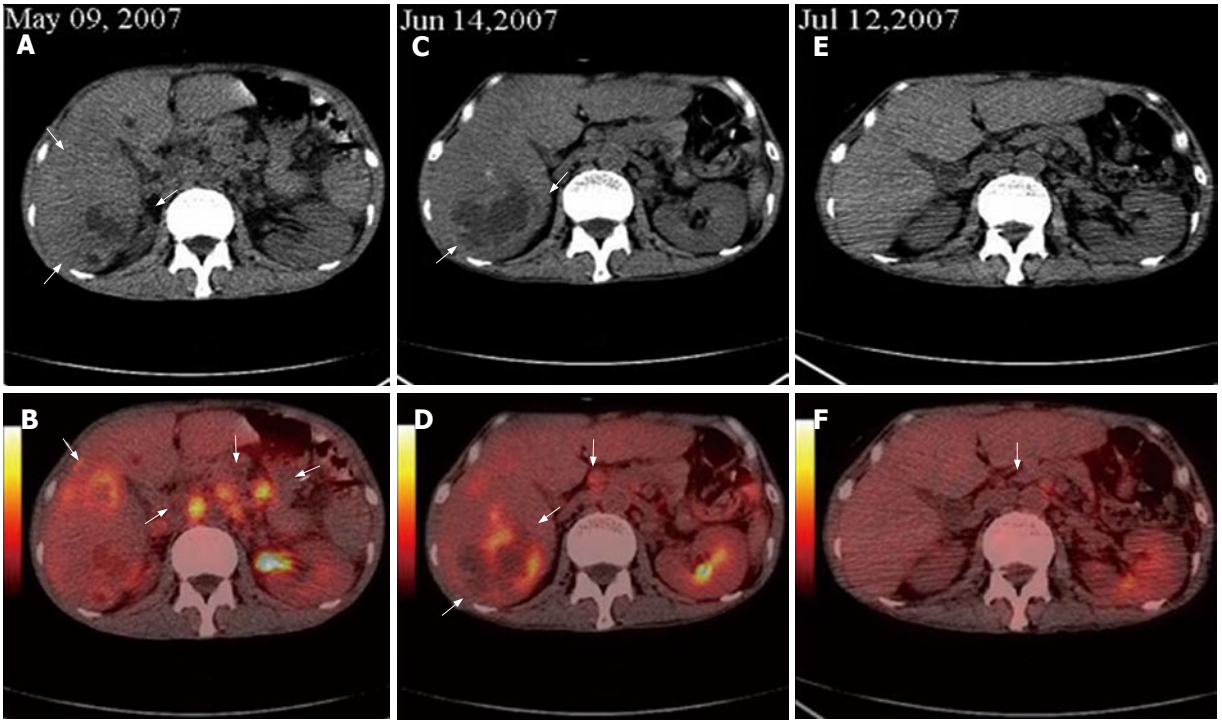

Figure 2 Base line FDG PET/CT detecting huge metastases in the right lobe of liver and highly metastatic lymphoid nodes in abdomen (white arrows, **A** and **B**), second FDG PET/CT demonstrating huge metastases in the right lobe of liver and the decreased number, size and metabolism level of metastatic lymphoid nodes (white arrows, **C** and **D**), third FDG PET/CT displaying the disappeared huge metastases in the right lobe of liver and the normal size of metastatic lymphoid nodes (**E** and **F**).

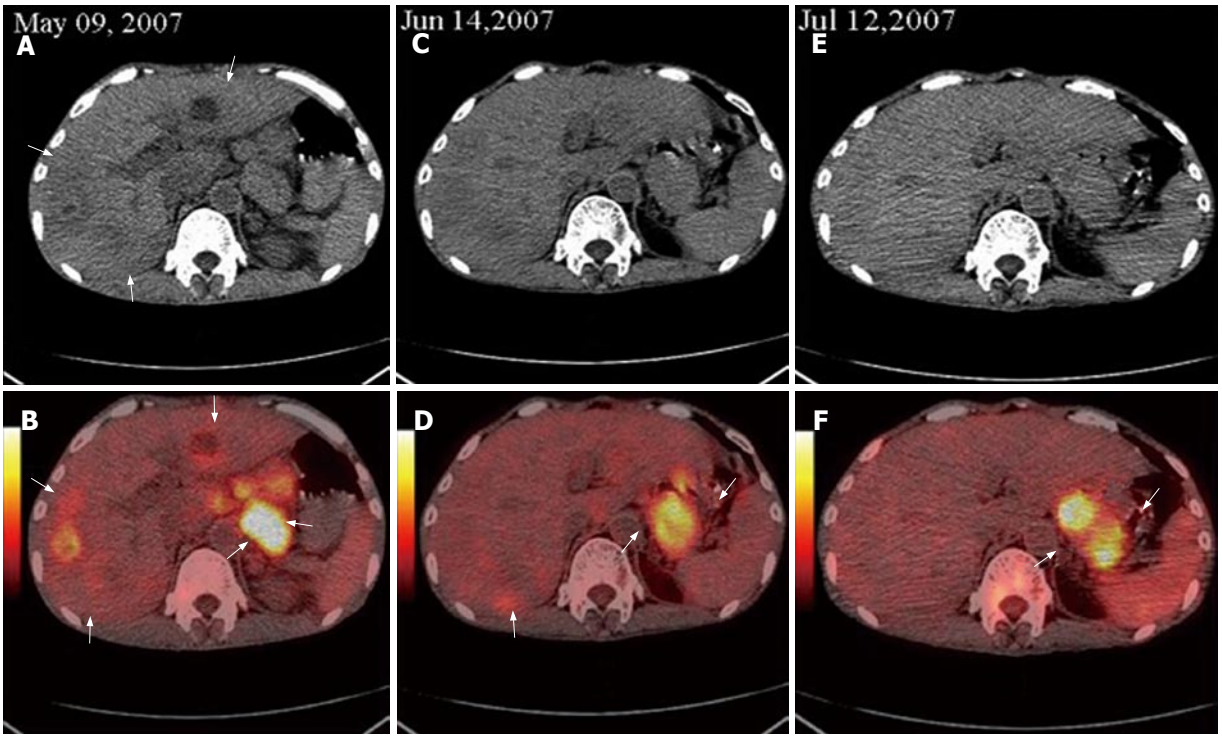

Figure 3 Base line FDG PET/CT detecting multi high metastases in both lobes of liver and highly metastatic lymphoid nodes in abdomen (white arrows, **A** and **B**), second FDG PET/CT demonstrating multi high metastases in the liver and the decreased number, size and metabolism level of metastatic lymphoid nodes (white arrows, **C** and **D**), third FDG PET/CT displaying disappeared liver metastasis and metastatic lymphoid nodes (**E** and **F**).

Cetuximab is a chimeric IgG1 monoclonal antibody and binds to EGFR with a high specificity and a higher affinity than either EGF or TGF-α, thus blocking ligand-induced phosphorylation of EGFR. Second line therapies with Cetuximab for colorectal cancer after failure of first-line regimes have shown a response rate of 17%-23% and an overall survival (median) of 8.6 mo[9]. Endostar is a new recombinant human endostatin developed by Medgenn

Bioengineering Co. Ltd (Yantai, Shandong, China) in 2005. A preclinical study indicated that endostar could inhibit tumor endothelial cell proliferation, angiogenesis and tumor growth, and results from prospective trials addressing the response of colorectal cancer to Cetuximab containing regimes have confirmed its efficacy in clinical practice[10].

Modern cancer care is critically dependent on imaging technologies, which are used to detect early tumors and guide their therapy or surgery[11]. Molecular imaging technologies provide information about the functional or metabolic characteristics of malignancies, tumor stage and therapeutical response, and tumor recurrence, whereas conventional imaging technologies predominantly assess the tumor's anatomical or morphologic features including its size, density, and shape, *etc*. Since conventional imaging technologies reveal morphology of lesions with nonspecific features, differentiation between malignant and benign lesions could be improved by molecular imaging. As our knowledge about the molecular basis of cancer increases, imaging methods that provide clinicians with telling details about the molecular environments of patients' tissues are needed. By using standard anatomic imaging technologies combined with molecular imaging technologies such as PET/CT, we can detect disease processes at the anatomic, physiologic, metabolic, and molecular levels, thereby, allowing earlier detection of diseases, monitoring of therapies, and better prognostication of disease progression[12].

The precise tailoring of treatment for patients with cancer is a challenge. PET/CT and SPECT/CT help achieve better results[13]. PET with the glucose analog ^{18}F-FDG is increasingly used to monitor the effectiveness of therapy for patients with malignant lymphomas and solid tumors. Quantitative assessment of therapy-induced changes in tumor ^{18}F-FDG uptake can predict tumor response and patient outcome early in the course of therapy. Treatment may be adjusted according to the chemosensitivity and radiosensitivity of tumor tissue in an individual patient. Thus, ^{18}F-FDG PET has a potential to reduce the side effects and costs of ineffective therapy[14]. The use of integrated PET/CT instead of PET in treatment monitoring poses some methodologic challenges against the quantitative analysis of PET scans. However, it may provide the opportunity to integrate morphologic and functional information. This integration may define new parameters for assessment of tumor response and facilitate the use of PET in studies as well as in clinical practice[14]. In our case, a partial response (> 30% reduction, according to RECIST) of hepatic metastasis and peritoneal carcinomatosis lesions was achieved after 4 wk of treatment with Cetuximab plus Endostar.

In conclusion, our case demonstrates that molecular imaging could monitor molecular treatment and combination of EGFR-specific antibodies with VEGF-specific antibodies may be a promising treatment modality. Further prospective trials are mandatory to confirm its effects and discriminate Cetuximab-induced response from Endostar-associated response. The potential of this novel approach to anticancer therapy should be elucidated in large clinical trials.

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Eosinophilic cholecystitis as a rare manifestation of visceral larva migrans

Hitoshi Yoshiji, Masahide Yoshikawa, Kosuke Kaji, Hiroshi Fukui

Hitoshi Yoshiji, Kosuke Kaji, Hiroshi Fukui, Third Department of Internal Medicine, Nara Medical University, Nara, Japan
Masahide Yoshikawa, Department of Parasitology, Nara Medical University, Nara, Japan
Correspondence to: Hitoshi Yoshiji, Third Department of Internal Medicine, Nara Medical University, Nara, Japan. yoshijih@naramed-u.ac.jp
Telephone: +81-744-223051 Fax: +81-744-247122
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Abstract

Eosinophilic cholecystitis is an infrequent form of cholecystitis. The etiology of eosinophilic cholecystitis is still obscure, and it is sometimes accompanied with several complications, but a simultaneous onset with pericarditis is very rare. We would like to make an alternative interpretation of our recent report "Kaji K, Yoshiji H, Yoshikawa M, Yamazaki M, Ikenaka Y, Noguchi R, Sawai M, Ishikawa M, Mashitani T, Kitade M, Kawaratani H, Uemura M, Yamao J, Fujimoto M, Mitoro A, Toyohara M, Yoshida M, Fukui H. Eosinophilic cholecystitis along with pericarditis caused by Ascaris lumbricoides: A case report. World J Gastroenterol 2007; 13: 3760-3762."

Key words: Cholecystitis; Visceral larva migrans; Parasite; *Ascaris suum; Toxocara canis*

Yoshiji H, Yoshikawa M, Kaji K, Fukui H. Eosinophilic cholecystitis as a rare manifestation of visceral larva migrans. *World J Gastroenterol* 2007; 13(45): 6119

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TO THE EDITOR

Eosinophilic cholecystitis is an infrequent form of cholecystitis. The etiology of eosinophilic cholecystitis is still obscure, and it is sometimes accompanied by several complications, but a simultaneous onset with pericarditis is very rare[1-3].

We would like to make an alternative interpretation of our recent report "Kaji K, Yoshiji H, Yoshikawa M, Yamazaki M, Ikenaka Y, Noguchi R, Sawai M, Ishikawa M, Mashitani T, Kitade M, Kawaratani H, Uemura M, Yamao J, Fujimoto M, Mitoro A, Toyohara M, Yoshida M, Fukui H. Eosinophilic cholecystitis along with pericarditis caused by Ascaris lumbricoides: A case report. *World J Gastroenterol* 2007; 13: 3760-3762." We reported that this rare clinical manifestation was caused by *Ascaris lumbricoides*. However, from the serological diagnosis, it was likely that these clinical symptoms were visceral larva migrans (VLM) caused by *Ascaris suum* or *Toxocara canis* rather than by *Ascaris lumbricoides*. While a definitive diagnosis of parasitic diseases is established after the detection of worms or eggs from the patient, the direct detection of the worm is quite rare. As we described in the report, we could not make a direct detection of the worm either. Generally, food-borne parasitic infection can be diagnosed based on the raw food intake history, laboratory data such as hypereosinophila, and the presence of antibody against the parasite in the serum. Our patient revealed a higher titer of antibody against *Ascaris suum* and *Toxocara canis* by enzyme-linked immunosorbant assay by 150 and 100 fold as compared with the control serum. Nevertheless, we can not rule out the possibility that it is caused by *Ascaris lumbricoides*. *Ascaris lumbricoides* also could penetrate the gastrointestinal tract.

In conclusion, since the title was too high in our study, we had to consider other alternative possibilities of VLM caused by *Ascaris suum* or *Toxocara canis* as described above, and we believe that this letter would help make our previous report more easily understood and legible for the readers.

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NEWS

Science news release and its benefits to your research

You-De Chang

You-De Chang, *World Journal of Gastroenterology*, The WJG Press, PO Box 2345, Beijing 100023, China
Correspondence to: You-De Chang, PhD, Science Editor, *World Journal of Gastroenterology*,The WJG Press, Room 1066, Yishou Garden, No. 58, North Langxinzhuang Road, PO Box 2345, Beijing 100023, China. y.d.chang@wjgnet.com
Telephone: +86-10-85381901 Fax: +86-10-85381893
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Abstract

News release to the latest science findings is beneficial to both researchers and their served institutions as well as the public. It will help to set a bridge of communication between researchers, the public and media, and publishers, making the latest research findings well known to the public. *World Journal of Gastroenterology* has currently freely opened the News Release Service System (*WJG*-NRSS) for original articles with potential significance and novelty for news release to mass media to broaden the findings to the public.

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http://www.wjgnet.com/1007-9327/13/6120.asp

News release to your research findings before publication in mass media is beneficial to both researchers and their served institutions. It will help to bridge the communication among researchers, the public and media, and publishers, particularly making the latest findings of the researchers well known to the public. An investigation from The New England Journal of Medicine shows that the citation rate of an article increase seven times when the findings of an article have been reported by New York Times.

World Journal of Gastroenterology (*WJG*) has currently freely opened the News Release Service System (*WJG*-NRSS) for the authorized reporters for news release in mass media such as EurekAlert!. As one of the registered members with identity of an international peer reviewed journal, *WJG* has been authorized to release science news on EurekAlert! (Figure 1).

As an online, global news service operated by AAAS, the science society, EurekAlert! is one of the central places through which *WJG* can bring the authors' latest findings to the media and public. *WJG* science news features the resources focused on all original articles with both significance and novelty. EurekAlert! has 923 registered entities including *WJG*, and has timely delivery of the released news to the public and its 5400 journalists worldwide.

Figure 1 *WJG* as a registered member to EurekAlert! with an identity of an international peer reviewed journal.

WJG has encouraged those articles with both significance and novelty to release news before its publication on EurekAlert! so that more readers could share the researcher's latest findings and ongoing research. The sample science news released by *WJG* on EurekAlert! are shown in Table 1. The number of hits shows that the four science news with amusive titles have been very attractive to readers.

An excellent news is very essential to achieve the expectations. In addition to basic guidelines for general news writings, we recommend some specific guidelines for our reporters in *WJG* science news writings. News titles may be different from the original article but should be attractive and informative. The main body of news is less than 1000 words with a summary of both less than 75 words. Interesting pictures are also strongly recommended. The deadline for news submission is about one week before publication. Since most readers to science news are not experts, journalists should avoid scientific jargon and use everyday language in news writings regardless of the topic or content. Finally but importantly, be sure the science news is lawful and ethical.

REFERENCES

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Table 1 Science news from *World Journal of Gastroenterology* released to EurekAlert!

Science news	Released media	Date posted	Hits since posted
Targeting nerve growth factor may cure liver cancer[1]	http://www.eurekalert.org/pub_releases/2007-09/wjog-tng090507.php	September 7, 2007	713
Researchers discover correlation between GERD and obesity in females[2]	http://www.eurekalert.org/pub_releases/2007-09/wjog-agr090407.php	September 5, 2007	756
Who will recover spontaneously from hepatitis C virus infection[3]	http://www.eurekalert.org/pub_releases/2007-08/wjog-wwr082807.php	August 29, 2007	504
Clearance of hepatitis C viral infection[4]	http://www.eurekalert.org/pub_releases/2007-08/wjog-coh082807.php	August 29, 2007	355

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2 **Piretta L**, Alghisi F, Anzini F, Corazziari E. Prevalence of overweightedness in patients with gastro-esophageal reflux *World J Gastroenterol* 2007; **13**: 4602-4605

3 **Wietzke-Braun P**, Manhardt LB, Rosenberger A, Uy A, Ramadori G, Mihm S. Spontaneous elimination of hepatitis C virus infection: a retrospective study on demographic, clinical, and serological correlates. *World J Gastroenterol* 2007; **13**: 4224-4229

4 **Ichikawa T**, Nakao K, Hamasaki K, Honda T, Shibata H, Akahoshi M, Eguchi S, Takatsuki M, Kanematsu T, Eguchi K. Clearance of hepatitis C virus after living-donor liver transplantation in spite of residual viremia on end date of interferon therapy before transplantation. *World J Gastroenterol* 2007; **13**: 4149-4151

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Golo Ahlenstiel, PhD, MD
Liver Diseases Branch - NIDDK, National Institutes of Health, 10 Center Drive, Bldg. 10, Room 9B11, Bethesda, MD 20892, United States

Taku Aoki, MD
Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

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VA New York Harbor Healthcare System, Division of Gastroenterology (111D), 423 East 23rd Street, New York, NY 10010, United States

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Department of Surgery, Catholic School of Medicine "Agostino Gemelli", Largo Agostino Gemelli 8 – 00168 Rome, Italy

Andres Cardenas, PhD
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Li-Fang Chou, Professor
Department of Public Finance, National Chengchi University, Chih-Nan Rd., Sec. 2, Zhi-Nan Road, Taipei 11623, Taiwan, China

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Department of Physiology and Biophysics, College of Medicine University of Arkansas for Medical Sciences, 4301 W Markham Street Little Rock, Arkansas 72205, United States

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Division of Research and Education, Scott and White Hospital and The Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

Inge Irma Depoortere, PhD
Centre for Gastroenterological Research, Gasthuisberg OandN, bus 701, Leuven 3000, Belgium

Radha K Dhiman, Associate Professor
Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Garcia-Compean Diego, MD, Professor
Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, N.L., Mexico

Jean Louis Frossard, PhD
Division of gastroenterology, Geneva University Hospital, Rue Micheli du Crest, 1211 Geneva 14, Switzerland

Xupeng Ge, MD, PhD
Transplant Biology Research Center, Massachusetts General Hospital, Harvard Medical School, MGH East, Building 149-5102, 13th street, Boston MA 02129, United States

Ajay Goel, PhD
Department of Internal Medicine, Division of Gastroenterology, Baylor University Medical Center and Charles A Sammons Cancer Center, 3500 Gaston Avenue, Suite H-250, Dallas, TX 75246, United States

Anna S Gukovskaya, Professor
VA Greater Los Angeles Health Care System ,University of California, Los Angeles, 11301 Wilshire Blvd, Los Angeles 91301, United States

Werner Hohenberger, Professor
Chirurgische Klinik und Poliklinik, Krankenhausstrasse 12, Erlangen D-91054, Germany

Aydin Karabacakoglu, PhD, Assistant Professor
Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Limas Kupcinskas, PhD
Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

Peter Laszlo Lakatos, MD, PhD, Assistant Professor
1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

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

Ton Lisman, PhD
Thrombosis and Haemostasis Laboratory, Department of Haematology G.03.550, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Peter J Mannon, MD
Mucosal Immunity Section, Laboratory of Host Defense, National Institute of Allergy, Laboratory of Clinical Investigation, Building 10/CRC, Room 6-3742, 9000 Rockville Pike, Bethesda, Maryland 20892, United States

Kevin McGrath, MD
Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center, M2, C wing, PUH, 200 Lothrop St, Pittsburgh, PA 15213, United States

James Neuberger, Professor
Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

Yaron Niv, Professor
Department of Gastroenterology, Rabin Medical Center, Beilinson Campus, Tel Aviv University, 2 Hadekel St., Pardesia 42815, Israel

Valerio Nobili, PhD
Liver Unit, Research Institute, Bambino Gesù Children's Hospital, S. Onofrio 4 Square, 00165 Rome, Italy

Luis Rodrigo, Professor
Gastroenterology Service, Hospital Central de Asturias, c/ Celestino Villamil, s.n., Oviedo 33.006, Spain

Francis Seow-Choen, Professor
Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10 , 228510, Singapore

Nick Paul Thompson, MD
Department of Medicine, Freeman Hospital, Newcastle Upon Tyne, NE7 7DN, Unite Kingdom

Adriana M Torres, PhD
Professor of Pharmacology, Suipacha 531, Rosario 2000, Argentina

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Meetings

Events Calendar 2007-2009

Meeting Falk Research Workshop: Morphogenesis and Cancerogenesis of the Liver
25-26 January 2007
Goettingen
symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Inflammatory Bowel Diseases 2007
1-3 March 2007
Innsbruck
ibd2007@come-innsbruck.at
www.come-innsbruck.at/events/ibd2007/default.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
Barcelona
easl2007@easl.ch
www.easl.ch/liver-meeting

Meeting SAGES 2007 Annual Meeting -part of Surgical Spring Week
18-22 April 2007
Paris Hotel and Casino, Las Vegas, Nevada
www.sages.org/07program/index.php

Meeting Falk Symposium 159: IBD 2007-Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
Washington-DC
tkoral@asge.org

Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
12-15 June 2007
Lisbon
fca@netvisao.pt

Meeting Falk Symposium 160: Pathogenesis and Clinical Practice in Gastroenterology
15-16 June 2007
Portoroz
symposia@falkfoundation.de

Meeting ILTS 13th Annual International Congress
20-23 June 2007
Rio De Janeiro
www.ilts.org

Meeting 9th World Congress on Gastrointestinal Cancer
27-30 June 2007
Barcelona
meetings@imedex.com

Meeting 15th International Congress of the European Association for Endoscopic Surgery
4-7 July 2007
Athens
info@eaes-eur.org
www.congresses.eaes-eur.org

Meeting 39th Meeting of the European Pancreatic Club
4-7 July 2007
Newcastle
www.e-p-c2007.com

Republic of meeting ISNM2007
The 21st International Symposium on Neurogastroenterology and Motility
2-5 September 2007
Jeju Island
isnm2007@intercom.co.kr
www.isnm2007.org/00main/main.htm

Meeting XXth International Workshop on Heliobacter and related bacteria in cronic degistive inflammation
20-22 September 2007
Istanbul
www.heliobacter.org

Meeting European Society of Coloproctology (ESCP) 2nd Annual Meeting
26-29 September 2007
Malta
info@escp.eu.com
www.escp.eu.com/index.php

18th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists
8-11 October 2008
Istanbul

Meeting Falk Workshop: Mechanisms of Intestinal Inflammation
10 October 2007
Dresden
symposia@falkfoundation.de

Meeting Falk Symposium 161: Future Perspectives in Gastroenterology
11-12 October 2007
Dresden
symposia@falkfoundation.de

American College of Gastroenterology Annual Scientific Meeting
12-17 October 2007
Philadelphia

Meeting Falk Symposium 162: Liver Cirrhosis-From Pathophysiology to Disease Management
13-14 October 2007
Dresden
symposia@falkfoundation.de

Meeting APDW 2007-Asian Pacific Digestive Disease Week 2007
15-18 October 2007
Kobe
apdw@convention.co.jp
www.apdw2007.org

15th United European Gastroenterology Week, UEGW
27-31 October 2007
Paris

Meeting The Liver Meeting®2007-57th Annual Meeting of the American Association for the Study of Liver Diseases
2-6 November 2007
Boston-MA
www.aasld.org

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Three-line tables should be numbered as 1, 2, 3 and so on, and mentioned clearly in the main text. Provide a brief title for each table. No detailed legend should be included under the tables. This part should be added into the text where the tables are applicable. The information should complement but not duplicate that contained in the text. Use one horizontal line under the title, a second under the column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

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Data that are not statistically significant should not be noted. $^{a}P<0.05$, $^{b}P<0.01$ should be noted ($P>0.05$ should not be noted). If there are other series of *P* values, $^{c}P<0.05$ and $^{d}P<0.01$ are used. Third series of *P* values can be expressed as $^{e}P<0.05$ and $^{f}P<0.01$. Other notes in tables or under

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English journal article (list all authors and include the PMID where applicable)

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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Organization as author

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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Present as mean ± SD or mean ± SE.

Statistical expression

Express t test as t (in italics), F test as F (in italics), chi square test as χ^2 (in Greek), related coefficient as r (in italics), degree of freedom as γ (in Greek), sample number as n (in italics), and probability as P (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p(B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2 not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

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Telephone: +86-351-4078656
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EDITORIAL

Endoscopic treatment of chronic pancreatitis

Laurent Heyries, Jose Sahel

Laurent Heyries, Jose Sahel, Hôpital de la Conception, 147 Bd Baille, Marseille 13005, France
Correspondence to: Jose Sahel, Service de Gastroentérologie, Hôpital de la Conception, 147 Bd Baille, Marseille 13005, France. jose.sahel@ap-hm.fr
Telephone: +33-4-91384021 Fax: +33-4-91752304
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Abstract

Treatment of chronic pancreatitis has been exclusively surgical for a long time. Recently, endoscopic therapy has become widely used as a primary therapeutic option. Initially performed for drainage of pancreatic cysts and pseudocysts, endoscopic treatments were adapted to biliary and pancreatic ducts stenosis. Pancreatic sphincterotomy which allows access to pancreatic ducts was firstly reported. Secondly, endoscopic methods of stenting, dilatation, and stones extraction of the bile ducts were applied to pancreatic ducts. Nevertheless, new improvements were necessary: failures of pancreatic stone extraction justified the development of extra-corporeal shock wave lithotripsy; dilatation of pancreatic stenosis was improved by forage with a new device; moreover endosonography allowed guidance for celiac block, gastro-cystostomy, duodeno-cystostomy and pancreatico-gastrostomy. Although endoscopic treatments are more and more frequently accepted, indications are still debated.

Key words: Chronic pancreatitis; Endoscopic treatment

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METHODS

Endoscopic treatment needs a team (operator, anaesthesiologist) aware with Endoscopic Retrograde Cholangiopancreatography (ERCP) procedures. Specific material is necessary: good fluoroscopy with the possibility to magnify pictures, and a duodenoscope with a 4.2 channel allowing insertion of high calibre stent (10 Fr). Moreover, a wide variety of endoscopic ancillary instruments is essential: metallic and hydrophilic guide-wire, sphincterotomes, Dormia basket, balloon dilatators and bougie dilatators (5-11.5 Fr), but also very thin guide wire (0.025 inches), fine-tipped sphincterotomes, Soehendra extractors (cf infra)[1]. Impaction of stones in pancreatic ducts needs the use of extracorporeal shock wave lithotripsy before endoscopic stone extraction[2]. Endoscopic treatment also needs naso-pancreatic drains and pancreatic stents that are either polyethylene or Teflon. The choice of length, pattern or external calibre of the stent is decided according to the anatomy of pancreatic ducts. Usually, straight stents with proximal and distal external flaps (to avoid internal or external migration), are used. Other stents like single or double pig-tail stents can be used also.

Endoscopic pancreatic sphincterotomy (PS)

Firstly described by Fuji[3], PS is generally performed as the first step in order to improve access to the pancreatic duct. A short (5-6 mm) sphincterotomy is oriented at 13 h with a pure section cutting. Narrowness of pancreatic ducts has justified using special device (thin 0.025 guide-wire, fine-tipped sphincterotome). Biliary sphincterotomy, which was firstly recommended before PS, seems not to be systematically performed because, contrary to the firstly experience, PS alone is not associated with a secondary biliary stenosis[4].

Complications of PS occur in 4.2%-12.6% of cases[4,5]. The morbidity rate also depends on other endoscopic procedures done at the same time such as pancreatic stenting or stricture dilatation. It also depends on the inclusion of patients presenting with recurrent attacks of pancreatitis secondary to sphincter of Oddi dysfunction. In this case, morbidity rate is higher, reaching 12.5%[6]. PS is probably no more or no less harmful than a biliary sphincterotomy which is associated with a morbidity rate of 5.4%-9.8% of cases[7-9]. Morbidity of PS seems also lower in case of post-PS drainage with a naso-pancreatic drain or a pancreatic stent[6]. In case of complete obstruction of the main pancreatic duct in the head, it is sometimes possible to access to the body of the pancreatic duct through the accessory papilla.

Treatment of pancreatic duct strictures by dilatation and stenting

The procedure consists in setting a 3-4 m length, hydrophilic-top guide wire deep inside the main pancreatic duct to realise a stricture dilatation with balloon or dilatators in order to insert a stent the calibre (5-10 Fr) and length (3-12 cm) of which depends on pancreatic duct anatomy. The length of the stent is adapted to bridge the stenosis (duct stricture and/or stone); the calibre of the stent depends on the highest calibre of dilatator successfully inserted through the duct stricture.

In practice, chronic pancreatitis duct strictures are more difficult to pass than biliary stenosis. They are usually associated with an impacted stone which may prevent deep insertion of the guide-wire into the main pancreatic duct. Moreover, pancreatic duct strictures are often narrow and tight because of expanding pancreatic fibrosis. For these reasons, a high rate of failure of dilatation of pancreatic stricture has been reported. Recently, a new technique of dilatation has been reported by Brand *et al*[1]. who used a device (*Soehendra extractor*) previously designed for the extraction of migrated biliary stents. Forage is realised by screwing this instrument through pancreatic duct stenosis on the guidance of a guide-wire. The morbidity rate is low (0%-13%) because forage is realized in a fibrotic area. Finally, among patients with a stricture which could not be passed with a 7 Fr dilatator, this device (7 Fr or 10 Fr) allowed to pass over the stenosis in all cases[1,10].

Plastic stents are clogged by lithostatine precipates, carbonate of calcium and bio bacterial film in a mean time of four to six mo[11,12]. Therefore, stents have to be retrieved or exchanged every four mo during a variable stenting length of time according to the main series (2-12 mo)[13]. Some authors prefer to leave the pancreatic stent and to exchange it only in case of recurrence of symptoms or infection but this strategy is associated with a risk of complications[14]. Metallic stent has been proposed because of a longer time of patency but those stents could also be completely obstructed by intra-luminar inflammatory granuloma with a risk of septic complication[15]. The morbidity rate of pancreatic stenting is widely varied in series. Early complications (before d 30 after ERCP) are distinct of late complications (> 30 d). The main early complication is acute pancreatitis (5%-39%), most of cases are benign, oedematous, spontaneously resolutive forms. A few cases of pancreatic abscess or cholangitis have been reported in the preliminary publications[15,16]. Late complications are stent-related: although stent migration is rare (5%), stent occlusion is very frequently encountered. Ductal lesions after stenting (dilatation, irregularity, stenosis) have been reported in 21%-80% of cases[17]. These lesions are associated with endosonographic parenchymal signs in 68% of cases[18]. In fact, in more than half of the cases, stenting-ductal lesions will regress four mo after retrieval of the stent[17].

Pancreatic stones extraction and lithotripsy

Pancreatic stones may be retrieved only after a previously PS. Since pancreatic stones are often impacted in the pancreatic duct upstream a duct stenosis, extraction of pancreatic stones is more difficult than extraction of biliary stones. Many difficulties have to be solved before: stenosis above stones have to be dilated, stones have to be fragmented with extracorporeal shock wave lithotripsy (ESWL). After good results obtained for biliary and kidney stones, ESWL has been firstly proposed for pancreatic stones in 1987[2]. There are three kinds of lithotripter generators: electro magnetic, electro hydraulic and piezo-electric. Stones are visualised under fluoroscopic or ultrasonographic guidance or both, treatment is realized in pro-cubitus position, under general analgesia or sedation.

Results of the major series are reported on Table 1.

Table 1 Results of extracorporeal shock wave lithotripsy

Authors	Patients (*n*)	Sessions (*n*)	Symptom free (%)	Fragmentation (%)	Clearance (%)
Sauerbruch[19]	24	1.6	37	87	50
Ohara[20]	32	4.6	79	100	75
Costamagna[21]	35	1.9	17	100	80
Delhaye[22]	123	1.8	53	99	59
Schneider[23]	50	2.4	76	85	56
Total	264	1.6-4.6	17-79	85-100	50-75

Without ESWL, complete clearance of pancreatic stones was less than 40%. ESWL is successful in 85%-100% of cases and wash-out of stones was obtained in 50%-75%. Pain disappeared in 17%-79% of cases[19-23]. Morbidity of ESWL is difficult to distinguish from the morbidity related to other endoscopic procedures. Nevertheless, main adverse events of ESWL are abdominal pain and attack of acute pancreatitis. Success factors are more dependant of the site than the size of the stone: juxta-papillary and main pancreatic duct stones are easier to extract than stones located in the tail or in the side branches. Large stones seem easier to break because easier to localize. Duct stenosis is often associated with a pancreatic stone and is a factor of recurrence of pain despite a complete clearance. Fragments of stones are retrieved through PS during a new ERCP, using an extractor-balloon or a Dormia basket; a naso-pancreatic drain is sometimes left to wash the pancreatic ducts during 48 h[22].

Endoscopic drainage of pancreatic cysts and pseudo-cysts

Drainage of pancreatic cysts is realised through the stomach wall (gastro-cystostomy) or duodenum wall (duodeno-cystostomy) in case of trans-mural drainage or through the papilla in case of trans-papillary drainage. The trans-mural way is dedicated to bulging cysts into the stomach or duodenum. A diathermic puncture is realized perpendicularly on the site of maximal bulge. After insertion of the catheter deep inside the cyst cavity, a sample of cyst fluid is taken for bacteriologic, biochemical and cytological analysis. A guide-wire is inserted in the cavity in order to realise multiple loops. Then, a careful cystostomy of 5-8 mm with a papillotome or by balloon dilatation is realised. Large cystostomies appear to be associated with a higher risk of haemorrhage than balloon dilatation. Finally, one or two double-pig-tail stents are inserted. In case of infected cyst or large amount of necrotic tissues which could occlude the stent, a naso-cystic drain for washing seems more adapted than a stent.

Trans-papillary drainage is dedicated to communicating cysts. After a selective cannulation of the pancreatic duct, a guide-wire deeply inserted and a pancreatic sphincterotomy, a dilatation of the tract between the pancreatic ducts and the cyst (a down-stream duct stenosis usually being associated), is performed. A stent is inserted into the pancreatic duct in order to bridge the area of communication between the ductal system and the cyst. The mean time of drainage is usually two mo but depends on the persistence of the cyst on the morphologic explorations[24]. In case of co-existence of pancreatic duct

Table 2 Endoscopic treatment of pancreatic duct stenosis

Authors	Patients (*n*)	Technical Success (%)	Early improvement of pain (%)	Follow-up (mo)	Long-term improvement of pain (%)
Grimm[38]	70	58	82	2-36	57
Cremer[14]	76	94	94	18-72	94
Ponchon[39]	33	85	74	12	52
Sauerbruch[40]	24	87	83	24	50
Delhaye[11]	123	95	100	14	37
Schneider[23]	50	86	70	20	70
Binmoller[41]	93		74	58	64
Smits[42]	51	96	81	64	24
Dumonceau[43]	70		95	24	95
Adamek[44]	80			40	54
Heyries[13]	70	85	62	29	58
Rösch[45]	1018	70	-	59	65
Total	1758	85	81	30	61

lesions, a long-term pancreatic stenting is necessary to prevent a recurrence of the cyst.

Endo-ultrasonography

Interventional endo-ultrasonography (EUS) is particularly interesting in three issues: treatment of pain by coeliac neurolysis, drainage of pseudo cysts and trans-gastric pancreatic drainage. Coeliac block is associated with a low morbidity (diarrhoea in 3.5% of cases)[25,26]. Treatment of pancreatic cysts under EUS guidance is dedicated for pseudo cysts which are not bulging in the gut[27-32]. In case of complete obstruction of the pancreatic duct, a pancreatico-gastrostomy can be achieved under EUS guidance[33,34].

RESULTS AND INDICATIONS OF ENDOSCOPIC TREATMENT

The aim of endoscopic treatment is improvement of pain. Analysis of the literature is difficult because (a) the variability of pain during the time and between patients[35-37] (b) the pain during CP is multifactorial: ductal or interstitial pancreatic hyperpression, inflammatory infiltration of peri pancreatic nerves ("pancreatic neuritis") or pseudo-cysts. Other complications of CP could also be associated with pain: duodenal stenosis, duodenal cystic dystrophy, biliary stenosis or duodenal ulcer[35]. Moreover, endoscopic methods are different: biliary sphincterotomy is not always associated with a pancreatic sphincterotomy, time of pancreatic stenting (two months to undetermined), or number of stents. In fact, there are several methods of treatment which aim to obtain a satisfactory drainage of a pancreatic and/or biliary duct.

Treatment of pancreatic pain

Drainage of pancreatic duct is reported in numerous articles[11,13,14,23,38-45]. Technical success was obtained in 85% of cases (58%-96%). Stents are left during variable length of time, from two mo to endless. Short-term improvement of pain was obtained in 81% of cases (62%-100%). After a follow-up of 30 mo (14-60 mo), improvement of pain dropped to 61% of cases (24%-95%). There was no clinical predictive factor of success despite of an early stage of CP reported in three series[13,41,43]. Communicating cyst and juxta-ampullary stenosis were the two reported morphological predictive factor of success. Surprisingly, stop of alcohol intake did not seem to modify results of the endoscopic treatment. Nevertheless, alcohol intake has to be stopped because morbidity and mortality of CP are more attributed to toxic habits (alcohol or tobacco) than to CP itself[46]. In cases of complete obstruction of pancreatic duct preventing access *via* the papilla, EUS-guided pancreatico-gastrostomy can be done. Results of this method are preliminary: a short series of four cases has reported good results in one case, recurrence of pain in two cases managed with another endoscopic treatment (stenosis of the pancreatico-gastrostomy in one case, disruption and spontaneous migration of the stent in the other case), and failure in one case (12 mo of follow-up)[33]. An additional factor of pain is peri-pancreatic inflammatory infiltrate of nerves: a prospective randomized comparison of endoscopic ultrasound and computed tomography-guided celiac plexus block has reported better results under EUS whereby an immediate improvement of pain occurred in 50% of cases but this result dropped to 30% after six months of follow-up. Efficiency appeared significantly more prolonged in the EUS-group and the ratio cost-efficiency was also better in this group[47]. More recently, a prospective study including 90 patients reported an immediate improvement of pain in 55% and in 10% after six months of follow-up[25]. Young age or previously pancreatic surgery were factors of poor results. Indication of celiac plexus block is limited in CP because of a relative immediate efficiency and especially a frequently recurrence of pain after six mo of follow-up (Table 2).

Treatment of pancreatic cysts and pseudo cysts

Evaluation of patient and collection are the first step to decide strategic therapy. Ultrasonography, computed-tomography, MRCP and EUS make it possible for clinician to determine the two major risks of endoscopic treatment which are haemorrhage and perforation. Haemorrhage depends on the presence of pericystic or peridigestive vessels, segmental portal hypertension and the haemorrhagic content of the cyst. Perforation depends on the distance between the digestive-wall and

Table 3 Results of endoscopic cysto-enterostomy during chronic pancreatitis

Authors	Cysto-gastrost	Cysto-duodenost	Failure (*n*)	Recurrence (*n*)	Secondarysurgery	Morbidity (*n*)	Mortality (*n*)
Dohmoto[48]	5	1	0	-	1	0	0
Cremer[49]	11	22	1	3	5	3	0
Bejanin[50]	9	5	2	3	6	3	0
Barthet[51]	12	66	0	14	2	12	1
Smits[52]	16	10	3	3	8	5	0
Binmoeller[53]	24		4	6	5	5	0
Total	181		10 (5.5%)	29 (16%)	27 (15%)	28 (15.5%)	1 (0.5%)

Table 4 Results of endoscopic transpapillary drainage of pancreatic cysts

Authors	Patients (*n*)	Stent (Fr)	Healing (*n*)	Recurrence (*n*)	Morbidity (*n*)	Secondary surgery (*n*)
Kozarek[55]	8	-	7	0	3	4
Dohmoto[48]	6	7	6	1	2	0
[1]Barthet[56]	30	7-10	23	3	4	5
[2]Smits[52]	19	7-10	14	-	1	-
Binmoeller[53]	37	5-7	35	5	1	-
Catalano[57]	21	5-10	17	1	1	2
Total (*n*)	121	7 Fr	102 (85%)	10 (9.8%)	12 (10%)	11 (11%)

[1]transpapillary drainage + cysto-gastrostomy ($n = 5$); + cysto-duodenostomy ($n = 5$); [2]transpapillary drainage + cysto-gastrostomy ($n = 4$); + cysto-duodenostomy ($n = 2$); + cysto-gastrostomy and cysto-duodenostomy ($n = 1$).

the cyst which should not exceed 10 mm. Results of transmural drainage were reported in six series between 1989 and 1992 including 191 cysts (Table 3)[48-53]. Mean rate of healing, failure and recurrence were respectively 78% (51%-82%), 5.5% (0%-16%) and 6.5% (3%-13%). Secondary surgical procedure was necessary in 14.9% of patients (12%-30%). Morbidity was 15.5% including, according to increasing rates of frequency, haemorrhage, perforation, and infection. Haemorrhage seems more frequent in case of gastro-cystostomy than for duodeno-cystostomy[51]. The only death reported concerned a patient who presented cirrhosis complicated by portal hypertension and associated with haemorrhagic pancreatic ascitis[51]. Long-term results of transmural drainage are not well-known, follow-up not exceeding 31 mo. A recent study including 34 patients followed 46 mo, reported good results in 62% of cases (in intent to treat) with only 71% of initially technical success[54]. Three cases of recurrence were reported, of whom two cases were successfully managed endoscopically.

Results of trans-papillary drainage have been also reported in six series from 1991 to 1995 including 121 cysts (Table 4)[48,53,55-57]. Symptom-free rates were 87% (76%-87%) and healing rates of cysts were 84% (76%-94%). Recurrence of cyst was 9.2%, morbidity was 10% with essentially septic complications and post-ERCP acute pancreatitis. A secondary surgical procedure was necessary in 10.8% of cases (9%-50%). Endoscopic drainage is intended for symptomatic cysts. In the other cases, drainage is necessary if the size of the cyst is more than 4 cm, particularly if the cyst is localized out of pancreatic area because, in this case, it uncommonly collapses spontaneously[58]. Trans-papillary drainage appears a first choice treatment in case of CP because pancreatic stent treats also pancreatic ductal lesions down stream the cyst and because it is less invasive than transmural way. Transmural drainage is especially reserved to large cysts but must be avoided in presence of segmental portal hypertension. Results of EUS-guided pseudo-cysts drainage have been recently reported in six series including 69 cases[27-32]. The most important monocentric study included 35 patients of whom 20 pancreatic abscess: after 27 mo of follow-up, drainage was successful in 94% of cases, a pneuperitoneum occurred in a case and has been managed conservatively, recurrence of cyst occurred in three cases of whom two abscess, surgical drainage was necessary in four cases of whom were four abscess. This method seems satisfying but has to be more evaluated in larger series[31].

Endoscopic treatment of biliary stenosis

Long-term results of biliary stenting have been reported in three series including 102 patients[59-61]. Although initial improvement was reported in 100% of cases, the rate of symptom free patients decreased to 17.5% (10%-28%) after 10 mo (14-49 mo) of follow-up; moreover, 68% of patients underwent a surgical bili-digestive diversion or were still stenting. Plastic stents temporarily improve cholestasis but are not able to dilate adequately common bile duct. In contrast with the previous series, a recent study including 25 patients using balloon dilatation before biliary stenting has reported excellent results in 80% of cases after 13 mo of follow-up[62]. These recent optimistic results attributed to the balloon dilatation, have to be confirmed. Morbidity is low (8%-9%) except migration or obstruction of the stent observed in respectively 14/25 and 18/25 patients of series of Deviere *et al* and Vitale *et al*[59,62]. Draganov *et al* tried to improve results by using several stents: nine patients underwent a biliary stenting with 2-($n = 3$) or 3-($n = 6$) 10 French-stent; 48 mo after

Table 5 Results of endoscopic biliary stenting of biliary stenosis associated with chronic pancreatitis

Authors	Patients (*n*)	Clinical improvement (%)	Stent obstruction (%)	Stent migration (%)	Healing (%)	Length of time of stenting (mo)
Devière [59]	25	100	32	40	12	-
Barthet[60]	19	100	0	5	10.5	10
Smits[61]	58	100	62	7	27	10
Vitale[62]	25	100	56		80	13

retrieval of stents, biliary stenosis recurred in only 55% of cases, absence of cephalic pancreatic stones was a factor of success[63]. Because plastic stent are not adapted for a long-term drainage, Deviere *et al* tested metallic expansive stent with an excellent result in 18/20 patients, after 33 mo of follow-up[64]. Nevertheless, the two remaining patients presented an obstruction of the stent secondary to epithelial hyperplasia in contact with the stent. More recently, a study including 13 patients presenting a biliary stenosis and unfit for surgical procedure, reported good results in 9/13 (69%) patients after 50 mo of follow-up, mean time of stent patency was 60 mo[65]. A single stent was enough in five cases, obstruction of the stent was managed by insertion of a plastic stent inside the metallic stent (n = 3) or by an extractor balloon (n = 1). In four (21%) cases, biliary drainage was not effective because of occluded stent (n = 3) or duodenal migration of the stent (n = 1). Three patients died for a cause not related to the biliary stenosis. Nevertheless, those metallic stents being not extractable, after a long-term follow-up, there is a possibility of stent occlusion by granulation reaction against a foreign body (Table 5).

Management of pancreatic exocrine and endocrine functions

Although improvement of pancreatic exocrine and endocrine functions is frequently discussed in surgical series, this notion is seldom reported in endoscopic series[13,22]. This is probably due to the difficulties to correctly explore the pancreatic exocrine function and also to the relatively short-term follow-up of endoscopic series in compared with surgical series. A temporary improvement of diabetes mellitus has been reported in 10% of cases after endoscopic treatment, while aggravation was noted in 12%[22]. Although another series reported an improvement in 26% of cases[13], most of the series did not noted any improvement[42,44]. Therefore, diabetes mellitus alone should not be an indication of endoscopic treatment of CP.

Evaluation of the effects of endoscopic treatment on pancreatic exocrine function is also seldom precise. A few studies report a gain of weight but this gain probably reflects more improvement of pain than improvement of exocrine function[13]. Evaluation of pancreatic exocrine function with a C^{14} breath test reports a 50%-60% improvement after endoscopic treatment[22].

Endoscopic treatment of pancreatic fistulas

Three major series included 39 patients presenting with a pancreatic fistula after acute pancreatitis (n = 19) and associated with CP (n = 12)[55,66,67]. Treatment consisted in a trans-papillary drainage of pancreatic duct in 34 cases, associated with a transmural drainage of a cyst in four cases. Rate of success was 92 %, with complications occurring in seven cases (17%). Complications included mainly acute pancreatitis and sepsis. Seven (17%) patients underwent surgical procedures 11 to 16 mo after endoscopic treatment. Few isolated cases of pancreatico-pleural fistula successful treated by endoscopic drainage, have been reported[68]. Trans-papillary drainage is also reported as a successful treatment for pancreatico-peritoneal fistulas.

ENDOSCOPIC TREATMENT AND SURGICAL PROCEDURES

Up to now, few randomised series have covered this topic. A recent study concludes in favour of surgery[69]. This study randomized 72 patients and after a follow-up of five years, although incomplete improvement of pain was equivalent in the two groups (46% *versus* 52%), a significant difference appeared for the complete resolution of pain (37% in the surgical group *versus* 14% in the endoscopic group). Nevertheless this series presents a bias because 80% of patients in the surgical group underwent a resection procedure and only 20% underwent a derivation procedure. Therefore, results of endoscopic treatment have to be compared with surgical procedure of derivation. Moreover, half of patients accepted randomization between surgery and endoscopy, this high rate of refusal emphasizes the difficulties in comparing the two methods and to set-up this kind of study. Nevertheless, more recently, another prospective series reported that surgical drainage of the pancreatic duct was more effective than endoscopic treatment[70]. Thirty-nine symptomatic patients having CP with distal obstruction of the main pancreatic duct and without inflammatory mass were randomized: 19 underwent endoscopic trans-papillary drainage (16 of whom also underwent ESWL) and 20 had operative pancreatico-jejunostomy. After 24 mo of follow-up, patients who underwent surgery had a significant ($P < 0.001$) lower pain score compared to endoscopic drainage. Moreover, complete or partial pain relief was achieved in 75% of patients of "surgical group" and only 36% of patients of "endoscopic group" ($P = 0.007$). Morbidity rate and length of hospital stay were similar in the two groups but there were more procedures in the "endoscopic group" than in "surgical group" (a median of 8 *vs* 3). To conclude, strategy depends on the expertise of the local teams, endoscopic treatment could be proposed as a first line treatment, before surgical procedure.

CONCLUSION

Endoscopic treatment of CP has certainly improved during the last two decades. Although results are clearly accepted as excellent for pancreatic cysts and pancreatic fistulas, long-term improvement of biliary and pancreatic ducts stenosis remains controversial.

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EDITORIAL

Smoking in inflammatory bowel diseases: Good, bad or ugly?

Peter Laszlo Lakatos, Tamas Szamosi, Laszlo Lakatos

Peter Laszlo Lakatos, Tamas Szamosi, 1st Department of Medicine, Semmelweis University, Budapest, Hungary
Laszlo Lakatos, 1st Department of Medicine, Csolnoky F. County Hospital, Veszprem, Hungary
Correspondence to: Peter Laszlo Lakatos, MD, PhD, 1st Department of Medicine, Semmelweis University, Koranyi str. 2/A, H-1083, Hungary. kislakpet@bel1.sote.hu
Telephone: +36-1-2100278-1500 Fax: +36-1-3130250
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Abstract

Smoking is an important environmental factor in inflammatory bowel disease (IBD), having different effects in ulcerative colitis (UC) and Crohn's disease (CD). A recent meta-analysis partially confirmed previous findings that smoking was found to be protective against ulcerative colitis and, after onset of the disease, might improve its course, decreasing the need for colectomy. However, smoking increases the risk of developing Crohn's disease and worsens its course, increasing the need for steroids, immunosuppressants and re-operations. Smoking cessation aggravates ulcerative colitis and improves Crohn's disease. Data are however, largely conflictive as well as the potential mechanisms involved in this dual relationship are still unknown. In this review article, the authors review the role of smoking in inflammatory bowel diseases.

Key words: Smoking; Crohn's disease; Ulcerative colitis; Phenotype

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INTRODUCTION

The pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD) has only been partly understood. Inflammatory bowel disease (IBD) is a multifactorial disease with probable genetic heterogeneity[1,2]. In addition, several environmental (eg, diet, smoking, measles or appendectomy) risk factors may contribute to its pathogenesis.

During the past decades, the incidence pattern of both diseases has changed significantly[3], showing some common but also quite distinct characteristics for the two disorders. Differences in geographic distribution, and particularly changes in incidence over time within one area, may provide insight into possible etiological factors[4]. It is very unlikely however, that these rapid changes attributed to variations in the genetic factors. On the contrary, environmental factors are likely to play an important role. Diet, as a luminal antigen, was thought to be an important factor in the pathogenesis of IBD[1,5]. In the last two decades, there has been a shift in the lifestyle in Eastern Europe, Asia, and Central America, as the lifestyle, including the diet, became more "Westernized". This possibility is further supported by the differences in incidence and prevalence found within one region.

A further important environmental factor studied extensively in both diseases is smoking. The link between smoking and (IBD) was first made in 1982 when Harries *et al*[6] noticed a low proportion of ulcerative colitis patients were smokers. Two years later a case-control study by Somerville *et al*[7] reported that the relative risk of developing Crohn's disease was 4.8 in those who smoked before disease onset, and 3.5 for those with a current smoking habit. In recent years, IBD has been classified into subtypes/phenotypes that are distinct and based on age at onset, disease location, and clinical behaviour. Knowledge of this heterogeneity has led to the re-examination of genetic and environmental influences on IBD. The relationship between smoking and IBD however, is far more complex than previously realized as clinical subtypes have become apparent. In this article, the authors give an updated review on the role of smoking in IBD.

EFFECT OF SMOKING CESSASION AND SMOKING ON THE RISK OF DEVELOPING IBD

Risk for developing ulcerative colitis

Ulcerative colitis affects predominantly non-smokers and former smokers. The percentage of current smokers (smoking more than seven cigarettes per week) in a group of patients with UC is about 10%-15%[8,9]. These percentages are significantly lower than those observed in a control population matched for sex and age (25%-40%). The meta-analysis by Calkins[10], conducted more than 15 years ago, yielded a pooled odds ratio of 0.41 (0.34-0.48) for current smokers compared with lifetime non-smokers. The effect of smoking seems to only postpone the event,

as the relative risk of UC was also higher in former smokers (OR: 1.64; 95% CI: 1.36-1.98). In a recent meta-analysis by Mahid *et al*[11] comparable values were reported, which also included new available data. Current smoking decreased the risk for UC (OR: 0.58; 95% CI: 0.45-0.75), while former smoking was associated with an increased risk (OR: 1.79; 95% CI: 1.37-2.34). Interestingly, in patients who stopped smoking, UC developed in 52% of patients, in the first three years after cessation, as reported by Motley *et al*[12] in concordance with other studies[13]. In contrast, active smoking in early childhood was associated with a gradually increased risk for developing UC (OR for smoking start < 10 years: 7.02 and < 15 years: 3.46)[14]. The same trend was observed for passive smoking by the mother (OR: 1.53, 95% CI: 0.93-2.49).

The relationship between smoking and ulcerative colitis has also been examined at a population level. The prevalence of UC was five-fold increased in patients from the Mormon Church in Britain and Ireland, where smoking is strongly discouraged, compared with that of the general population. In contrast, CD was equally as common[15]. In addition, a review of 56 epidemiological studies in Sweden over the time period from 1930 to 1990[16] demonstrated that the sex distribution of UC had changed from an earlier female predominance to a later male predominance. Over the same period, the proportions of smokers and ex-smokers among men and women have undergone reciprocal changes with an increase in women smokers relative to men, while the same change in predominance was not observed in contemporary pediatric studies. Somewhat contradictory, in a recent population-based case-control study[17], among others, ever smoking was also associated with increased risk (1.66; 95% CI: 1.17-2.35).

There are only very few data regarding the effect of smoking in indeterminate colitis[8,18]. The effect seems to be similar to that observed in UC, that is, a protective effect against the development of colitis and a possible beneficial effect on disease course.

Risk for developing Crohn's disease

The percentage of current smokers in a group of patients with CD is significantly higher than that observed in a control population matched for sex and age (45%-55% *vs* 30%-40%)[19]. In concordance, an increased life-time risk was reported in current smokers when compared with non-smokers by both Calkins *et al*[10] (OR: 2.0; 95% CI: 1.65-2.47) and in the more recent meta-analysis by Mahid *et al* (OR: 1.76; 95% CI: 1.40-2.22).

Compared with never-smokers, former smokers were reported to have an increased risk of developing CD[10]. This risk decreased only after four years of having quit smoking. In a recent population-based study by Bernstein *et al*[19], similar data were reported, both current smoking (OR: 1.96) and ever smoking (OR: 1.78) were associated with increased risk to develop CD. However, this later association could not be replicated in the recent meta-analysis by Mahid *et al* although a trend was observed ($P = 0.08$). In contrast, ever smoking was associated with increased risk (OR: 1.61; 95% CI: 1.27-2.03). The effect of passive smoking remains controversial[20]. In one recent prospective study[14] CD patients were more likely than controls to have prenatal smoke exposure (OR: 1.72; 95% CI: 1.1-2.71). In addition, the passive smoke exposure during childhood, with parents or other household members being smokers (OR: 2.04; 95% CI: 1.28-3.31) was also associated with increased risk, in concordance with previous data by Lashner *et al*[21].

EFFECT OF SMOKING AND ITS CESSATION ON DISEASE PHENOTYPE AND COURSE

Effect of smoking and its cessation on clinical course and extent of ulcerative colitis

Although the extent at diagnosis is not affected by smoking, UC usually runs a more benign disease course in smokers compared with non-smokers. Flare-up, hospitalization rates[13], the need for oral steroids[22] and colectomy rates[22,23], are reported to be lower, while age at onset is older in smokers compared with non-smokers, though not in all studies. Relapse rates are lower in patients who began smoking after the diagnosis of UC[24]. In concordance, in a recent Europe-wide population-based cohort[25] the relapse rate was lower (Hazard Ratio: 0.8; 95% CI: 0.6-0.9) in smokers compared with non-smokers, while it was higher in women. In a retrospective analysis of a large series of patients with UC, current smoking was found to decrease the 10-year cumulative colectomy risk from 0.42 to 0.32[22]. In concordance, a meta-analysis of several large series with a total of 1489 UC patients also found the risk for colectomy to be lower (OR: 0.57; 95% CI: 0.38-0.85) in current smokers compared with non-smokers[26].

In addition, in smokers with distal UC at diagnosis, the proximal extension of the disease is less frequent[22,27], while primary sclerosing cholangitis is observed almost exclusively in non-smokers[28]. Disease regression[29] was also more likely to occur in smokers compared with non-smokers or ex-smokers 5 years (30% *vs* 5% *vs* 8%), but not 10 years after the diagnosis. Also, those with extensive disease were the lightest smokers, whereas those with healthy colons were the heaviest smokers. Finally, current smokers have a lower incidence of pouchitis following coloproctectomy with ileal reservoir when compared with non-smokers[30,31].

In contrast, intriguing new data by Aldhous *et al*[29] showed that current and non-smokers had an almost identical age at onset (31.1 *vs* 29.4 years) and this was delayed only in ex-smokers (46.5 years). Colectomy rates were not different. This group however, had a greater exposure to smoking compared with the group of current smokers.

A link between smoking habits and the course of UC has also been reported. In intermittent smokers, many patients note symptom exacerbation when they stop smoking, followed by symptom relief when they smoke again[12]. In contrast, almost half of the intermittent smokers thought that their colitis symptoms improved while smoking at least 20 cigarettes per day[32]. Moreover, smokers with UC who quit, experience an increase in

disease activity, hospital admissions, and the need for major medical therapy (oral steroids, immunosuppressants), within the first years following the cessation of smoking[33]. However, the risk of colectomy in the short-term was not increased compared with matched non-smokers and continuing smokers.

Effect of smoking and its cessation on disease location, behaviour, and disease progression in Crohn's disease

Smoking is associated with disease location: most, but not all, studies report a higher prevalence of ileal disease and a lower prevalence of colonic involvement in smokers[34-36].

A recent review[36] and previous data have demonstrated that smoking, when measured up to the time point of disease behavior classification, was associated more frequently with complicated disease, penetrating intestinal complications[34,37,38], and greater likelihood to progress to complicated disease, as defined by development of strictures or fistulae[36], and a higher relapse rate[2,39]. Of note, previous severity of the disease, as assessed from the therapeutics needs, was found to be similar in young patients who started smoking and in their matched controls[10]. The need for steroids and immunosuppressants is increased in smokers compared with non-smokers[35]. Whether the daily dose (eg, more than 15 cigarettes per day) or the total pack years smoked is more important in the abovementioned associations remains questionable.

The risk of surgery as well as the risk for further resections during disease course is also higher in smokers, in most studies[34,41,42]. These findings were reinforced by Cottone *et al*[43] who have shown that macroscopic lesions on the ileal site of the anastomosis were observed 1 year after surgery in 70% of smokers, *versus* 35% of non-smokers and 27% of ex-smokers. The risk of symptomatic postoperative recurrence was more marked in heavy smokers than in mild smokers[43]. Noteworthy, immunosuppressive therapy was found to neutralize the effect of smoking on the need for surgery[40].

However, the harmful effect of smoking on the course of CD is not a universal finding. Studies in patients from Israel and Hungary have not found differences in the need for surgery or for immunosuppressants between smokers and non-smokers[2,44,45], and patients with only colonic involvement are less sensitive to the harmful effects of smoking[8]. Finally, the development and severity of perineal complications do not seem to be influenced by the smoking status[39].

In a recent paper by Aldhous *et al*[46] using the Montreal classification, the harmful effect of smoking was only partially confirmed. Although current smoking was associated with less colonic disease, the smoking habits at diagnosis were not associated with time to development of stricturing disease, internal penetrating disease, perianal penetrating disease, or time to first surgery. Age at diagnosis was also similar in current smokers and non-smokers (28.3 years and 28.9 years) and was only delayed in ex-smokers (43.2 years). However, the way in which they measured tobacco exposure was different from previous studies. "Current smokers" were defined as those who were smoking at the time of diagnosis or event (penetrating or stricturing complication) and "ex-smokers" had stopped for at least one year before the diagnosis or event (eg, a patient could have continued smoking up to 1 year before developing a complication but was considered an "ex-smoker").

The rationale for this may be that after surgery, the risk of endoscopic and clinical recurrence in former smokers who have not smoked for at least 1 year is similar to that of non-smokers[43]. Similarly, CD activity in ex-smokers is not different from that of non-smokers, and is less marked than in current smokers[39]. The beneficial effect of quitting smoking might be seen within the year following cessation. A large prospective intervention study by Cosnes *et al*[47] performed in a selected group of 59 patients who stopped smoking following a smoking cessation intervention, examined the disease course from 1 year following smoking cessation onwards. The flare-up rate, therapeutic needs, and disease severity were similar in patients who had never smoked and in those who stopped smoking, and both had a better course than current smokers. Quitters had a 65% lower risk of flare-up compared with continuing smokers. The need for corticosteroids, immunosuppressive therapy, or a dose increase of immunosuppressants was also lower. Interestingly, after quitting, some patients developed UC-like lesions of the distal colon, whereas previously they had typical CD.

Finally, in a prospective study during pregnancy, the improvement of disease activity was observed only in smokers, in parallel with a decrease in the daily cigarette smoking[48].

The role of gender, familial disease, and ethnicity

The effect of smoking is to some extent different between male and female patients. In CD, women are affected more drastically by smoking. The relative risk associated with smoking for women may be greater than for men: one study demonstrated a three-fold difference[20].

This was already demonstrated by Sutherland *et al*[42] in 1990, who reported that in a group of 174 patients who required surgery for Crohn's disease, smokers had a 29% greater risk than non-smokers, over 10 years. However, the increased risk was more marked in females than males (OR: 4.2; 95% CI 2.0-4.2 in females and 1.5; 95% CI 0.8-0.6 in males). In Crohn's colitis, smoking is clearly harmful for women, whereas colitis in men is not affected by smoking[8]. In the study by Cosnes *et al* current smoking hastened disease onset (from 35 to 29 years of age) and increased the need for immunosuppressants, only in women.

In UC, current and ex-smoking delayed disease onset in men (from 25 to 42 years of age), but not in women[50]. Similarly, when compared with non-smokers, male UC patients who smoked ran a more benign disease course as assessed by the decreased need for immunosuppressive therapy (8% *vs* 26%), whereas this difference was not observed in females as reported by Cosnes *et al*[8] and again smoking delayed the onset of disease, only in males.

Thus, the effect of smoking in both UC and CD seems to be modulated by gender, with women being affected more disadvantageously than men. This phenomenon deserves even more attention, since smoking habits are changing in the Western population, with a trend to a greater prevalence of smokers among young women[50].

Table 1 Smoking in IBD: Practice points

Ulcerative colitis (UC)	Crohn's disease (CD)
Current smoking decreases the risk for UC by app. 50%, in contrast former smoking is associated with an app. 2-fold increased risk	Both current and former smoking (presumable also passive smoke exposure during childhood) increases the risk of CD almost 2-fold
The protective effect is smaller in females	The risk is greater in females compared with males
Proximal extension of the disease is less likely in smokers as well as disease course is milder but the risk of lung cancer and vascular disease is higher	Smoking is associated with complicated (stricturing or penetrating) and ileal disease
Patients who stop smoking experience an increase in disease activity at least during the first year after cessation	Smokers with CD need more steroids, more immunosuppressants and more operations than non-smokers
The effect of smoking is similar in indeterminate colitis (less evidence is available)	Smoking cessation improves rapidly the course of CD
Nicotine-replacement therapies and antidepressants are useful in heavy smokers motivated to stop smoking	
Geographic differences exists (e.g. Israel, Korea)	

A gender difference in smoking habits may be a possible explanation, since women use more filtered cigarettes and lighter cigarettes, and might consequently have a higher relative exposure to smoke than to nicotine[51]. In addition, the negative effect of estrogens[52] on proinflammatory cytokine gene regulation and lymphocyte interactions might also play an important role.

In Israel, no association was reported between smoking and CD in Jewish patients, although the opposite association was found for UC[53]. Similarly, smoking was not associated to the risk for CD in some studies from Asia[54]. The reason why CD in Israeli Jews or Koreans is not as sensitive to smoking as in other populations is not clear. Explanations might include differences in genetic and/or environmental factors (eg, type of tobacco, way of smoking or other alimentary factors).

A further interesting question is whether the effect of smoking is similar in familial versus sporadic cases. Family studies have reported a high concordance rate within families, between smoking habits and the phenotype of IBD, CD developing in smokers, and UC in non-smokers[55]. The above association was refined in more recent studies. Tuvlin *et al*[56] reported that ex-smokers made up an increasing percentage of older patients diagnosed with UC, accounting for more than 35% of the attributable risk of late onset (> 45 years) UC and a large component of the second peak in diagnosis. In contrast, current smoking accounted for a large percentage of patients diagnosed at a younger age with familial CD but not with sporadic CD (Table 1).

MECHANISMS BEHIND THE EFFECT OF SMOKING ON IBD

The reason behind the opposite effects of smoking observed in CD and UC remain obscure. The effects of smoking and nicotine are numerous and since the pathogenesis of inflammatory bowel disease is only partially understood, any discussion on the possible mechanisms can only be speculative. In addition, although nicotine is thought to be the most important agent responsible for the effect of smoking, one should be careful, as some effects usually associated with smoking may not follow the use of therapeutic nicotine formulations. Of note in most studies, side-effects (nausea, headache, dermatitis) of nicotine therapies were frequent and tended to be greater than the clinical benefit[57].

Smoking has numerous specific and non-specific effects. It has been shown to affect the immune system, by influencing cellular and humoral immunity. Nicotine has been shown to decrease the synthesis of proinflammatory molecules, for example, interleukin (IL)-1β and TNFα by mouse colonic mucosa as well as the production of mucosal eicosanoids[58] and some proinflammatory cytokines by human mononuclear cells (eg, IL-2[59], IL-8, and TNFα[60]) partly by its action on the nicotinic acetylcholine receptor a7 subunit. Further evidence for anti-inflammatory properties comes from a surgically-induced ileus model, where carbon monoxide-treated mice were shown to have three times higher levels of IL-10. Macrophages from smokers express a selective functional deficiency in their ability to kill intracellular bacteria[61]. Finally, chronic exposure of rats to nicotine inhibits the antibody-forming cell response, impairs the antigen-mediated signaling in T-cells and induces T-cell anergy[62]. Other effects of nicotine or smoking on the intestine include the alteration of gut motility, the reduction of smooth muscle tone and contractility (modulated by nitric oxide)[63], decreased permeability[64], and alterations in the microcirculation[65], Furthermore, smoking also increases lipid peroxidation.

In UC, the colonic mucosal layer is thin or absent, in contrast to CD where it is significantly thicker[66], with nicotine having been shown to increase mucin synthesis[67,68]. Smokers with IBD have a significant reduction in mucosal cytokine levels, specifically, IL-1b and IL-8 in patients with UC, and IL-8 in patients with CD[69]. Beneficial effects of nicotine in active UC may be associated with a decrease in IL-8 expression. Hypoperfusion of the rectum and of acutely damaged colonic tissue may play an additional role[70]. On the contrary, in CD, several plasma antioxidant parameters are altered, the total radical-trapping antioxidant potential is decreased[71], and abnormalities are present in the microvasculature[65]. Smoking through increased carbon monoxide concentration might amplify the impairment in the vasodilation capacity of chronically inflamed microvessels, resulting in ischemia and perpetuating ulceration and fibrosis[70]. Furthermore, smoking is known to increase the thrombotic potential associated with vascular damage. A defect in bacterial clearance or macrophage deficiency might also play an important role.

CONCLUSION

In conclusion, smoking plays a dual role in IBD by increasing the risk for CD and decreasing that of UC. In an individual who is genetically at risk for IBD, smoking might be an important factor in determining disease phenotype. In addition, smoking also affects the disease course. It improves UC and worsens CD, more markedly in women, while smoking cessation is followed rapidly by a reversed effect. Since smoking is associated with several additional deleterious effects (eg, cardiovascular, lung cancer risk), gastroenterologists should encourage both UC and CD patients to quit smoking. Before stopping, UC patients should be informed about the potential risk of increase in disease activity, without a higher risk for surgery. In CD, the benefit of smoking cessation is well-proven, since patients who continue to smoke have a more severe course of disease with more complications, while ex-smokers run a similar course of disease to non-smokers.

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REVIEW

Small bowel capsule endoscopy in 2007: Indications, risks and limitations

Emanuele Rondonotti, Federica Villa, Chris JJ Mulder, Maarten AJM Jacobs, Roberto de Franchis

Emanuele Rondonotti, Federica Villa, Roberto de Franchis, Università degli Studi di Milano, IRCCS Fondazione Policlinico, Mangiagalli, Regina Elena.Unità Operativa di Gastroenterologia 3 via Pace 9, Milano 20122, Italy
Chris JJ Mulder, Maarten AJM Jacobs, VU University Medical Centre, Department of Gastroenterology and Hepatology, Boelelaan 1117, 1081 HV Amsterdam, The Nederlands
Correspondence to: Emanuele Rondonotti, MD, Universutà degli Studi di Milano, IRCCS Fondazione Policlinico, Mangiagalli, Regina Elena, Unità Operativa di Gastroenterologia 3, Via Pace 9, 20122 Milano,
Italy. emanuele.rondonotti@unimi.it
Telephone: +39-2-55035332 Fax: +39-2-50320747
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Abstract

Capsule endoscopy has revoluzionized the study of the small bowel by providing a reliable method to evaluate, endoscopically, the entire small bowel. In the last six years several papers have been published exploring the possible role of this examination in different clinical conditions. At the present time capsule endoscopy is generally recommended as a third examination, after negative bidirectional endoscopy, in patients with obscure gastrointestinal bleeding. A growing body of evidence suggests also an important role for this examination in other clinical conditions such as Crohn's disease, celiac disease, small bowel polyposis syndromes or small bowel tumors. The main complication of this examination is the retention of the device at the site of a previously unknown small bowel stricture. However there are also some other open issues mainly due to technical limitations of this tool (which is not driven from remote control, is unable to take biopsies, to insufflate air, to suck fluids or debris and sometimes to correctly size and locate lesions).The recently developed double balloon enteroscope, owing to its capability to explore a large part of the small bowel and to take targeted biopsies, although being invasive and time consuming, can overcome some limitations of capsule endoscopy. At the present time, in the majority of clinical conditions (i.e. obscure GI bleeding), the winning strategy seems to be to couple these two techniques to explore the small bowel in a painless, safe and complete way (with capsule endoscopy) and to define and treat the lesions identified (with double balloon enteroscopy).

Key words: Capsule endoscopy; Double balloon enteroscopy; Obscure glycemic index bleeding; Crohn's disease; Nonsteroidal anti-inflammatory drugs

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INTRODUCTION

The small bowel (SB) has been considered for a long time technically difficult to evaluate for many anatomical (i.e. distance from external orifices, length) and physiological (i.e. active peristalsis) reasons.

Until the introduction of Video Capsule Endoscopy (VCE) in clinical practice the small bowel was studied mostly with radiological or nuclear medicine techniques such as abdominal Computed Tomography (abdominal CT), abdominal Magnetic Resonance Imaging (abdominal MRI), small bowel follow through (SBFT), small bowel enteroclysis (SB enteroclysis) and 99_{m}TC scan. Although CT scan and abdominal MRI are highly sensitive in recognizing the presence of abdominal masses and allow an accurate evaluation of solid organs, lymph nodes and vessels, they are able to provide limited information about the small bowel wall. On the other hand, small bowel follow-through and small bowel enteroclysis, although specifically designed to evaluate the small bowel, have low sensitivity and specificity in recognizing small and flat lesions[1].

Additionally these two techniques are often poorly tolerated by patients and sometimes difficult to interpret.

The endoscopic evaluation of the small bowel represents the best possible approach to small intestinal diseases, allowing a direct visualization of small bowel mucosa, the collection of targeted biopsies and sometimes an effective treatment. Sonde enteroscopy, introduced because of its theoretical capability to visualize the entire small bowel (achievable in about 80% of examinations in clinical practice)[2,3], had been abandoned at the end of the 90's because of several technical limitations (angulation of the tip due to the presence of the balloon, duration of the examination, patient discomfort, inability to take biopsies)[4]. Push Enteroscopy (PE) is limited by the depth of insertion of the instrument to the proximal jejunum (about 90-150 cm from the oral route) and to

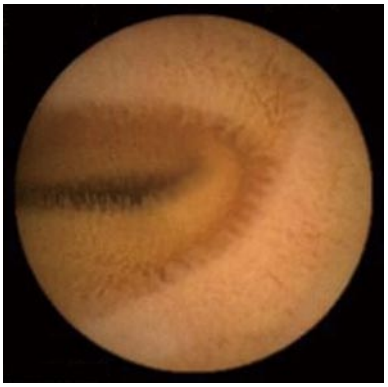

Figure 1 Normal small bowel.

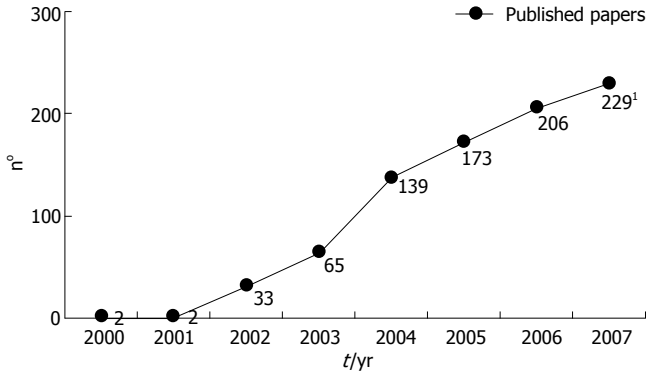

Figure 2 Published papers about capsule endoscopy between 2001 and 2007 (search engine: www.pubmed.org, key words: Capsule endoscopy OR capsule enteroscopy). [1]Estimated number of published articles in 2007 based on the number of papers published in the first seven months of the year.

the terminal ileum (50-80 cm in the retrograde way) and, despite sedation, is still poorly tolerated[5-7]. Intraoperative enteroscopy (IOE) is the most complete but also the most invasive means of examining the small bowel[7].

The introduction (in 2001)[8] and further continuous development of capsule endoscopy opened a new chapter in the study of small bowel diseases allowing, finally, to cross the frontier of the endoscopic examination of the small bowel. In fact this revolutionary technique made it possible, for the first time, to obtain high resolution endoscopic images of the entire small bowel (Figure 1) avoiding sedation, surgical intervention or radiation exposure. Capsule endoscopy showed, in everyday clinical practice, that the small bowel can be involved in several diseases (i.e. inflammatory, vascular, neoplastic, iatrogenic diseases). The knowledge of the large spectrum of lesions and diseases that can affect the small bowel stimulated the development and/or the implementation of other diagnostic and therapeutic techniques such as double balloon enteroscopy (DBE), MRI-enteroclysis and CT enteroclysis.

Performing a recursive search in the literature (by means of the most common search engine www.pubmed.org; using "capsule endoscopy OR capsule enteroscopy" as key words) we found a number of papers, increasing over the years, up to 754 (Figure 2). On the one hand this phenomenon certainly represents a proof of the revolutionary potential of this diagnostic tool in the field of small bowel endoscopy and, on the other hand, demonstrates the effort to establish the appropriate role of this device in different clinical conditions. Unfortunately about a quarter of published papers are case reports (187) or collections of small case series and 131 published papers are expert reviews. Following the rules of evidence based medicine[9] we can classify these papers at the lowest level of scientific evidence while, among the huge number of publications about capsule endoscopy, there are only 8 randomized controlled studies [five about bowel preparation, 2 about Nonsteroidal anti-inflammatory drugs (NSAIDs) induced damage and 1 about obscure GI bleeding (OGIB)][10-17] and 4 metanalyses (about OGIB or Crohn's disease)[18-21]; all these papers can be ranked as evidence grade 1c.

Mainly on the ground of the former 12 mentioned papers three Practice Guidelines have been published so far, two (in 2004 and 2006) on behalf of the European Society of Gastrointestinal Endoscopy (ESGE) and one on behalf of the American Society of Gastrointestinal Endoscopy (ASGE)[22-24] in 2006.

The aim of the present paper is to briefly review the evidence, available to date, about the use of capsule endoscopy for the study of the small bowel (starting from the studies providing the highest grade of evidence), to highlight the benefits of this technique but also to highlight risks and limitations which have emerged in these six years of use of the device in clinical practice.

INDICATIONS

Obscure GI bleeding

So far, OGIB is the main clinical indication for capsule endoscopy: about 70%-80%[25,26] of patients undergoing capsule endoscopy suffer from OGIB. The majority of studies published at the beginning of the experience with this new tool reported a high, although widely variable, diagnostic yield (ranging between 38% and 93%, about 75%-80% in most studies[27]). These studies, mainly performed in tertiary referral centres, collected highly selected patients with a long standing history of obscure GI bleeding, with low levels of haemoglobin at the time of the examination, who had undergone a huge number of prior examinations with negative results[28]. Subsequent studies performed on larger populations of patients, similar to those undergoing this examination in everyday clinical practice, showed a slightly lower diagnostic yield (about 50%)[29].

Although recent studies showed a decrease in the diagnostic yield of capsule endoscopy, two metanalyses[18,19] clearly demonstrated that capsule endoscopy in patients with obscure GI bleeding is superior to traditional radiological techniques (SBFT and SB enteroclysis) and PE. The latter comparison has been also recently confirmed in a specific prospective randomized controlled study[11]. The authors hypothesized that the high diagnostic yield of capsule endoscopy in this subgroup of patients may depend on the capability of the capsule to evaluate the mid-distal small bowel (particularly in comparison with PE) and/or to show small and flat lesions (vascular-

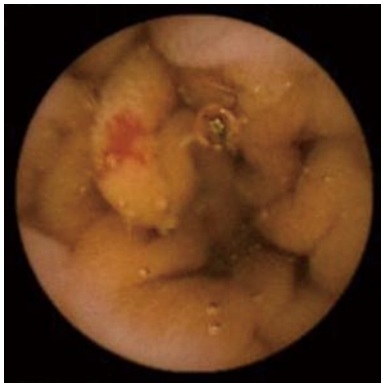

Figure 3 Artero-Venous Malformation (AVM) of the distal duodenum in a patient undergoing capsule endoscopy for obscure GI bleeding.

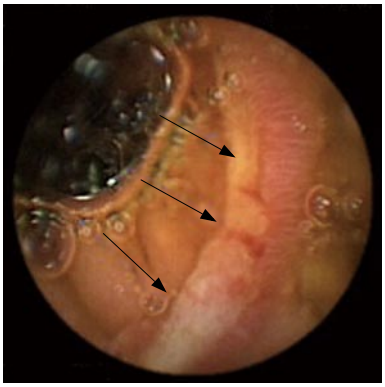

Figure 4 Jejunal ulcers in a patient with Crohn's disease.

Figure 3- or inflammatory-Figure 4) that are often missed by conventional radiological techniques.

As far as the possible factors potentially affecting the diagnostic yield of capsule endoscopy are concerned, the presence of active bleeding at the time of examination[30] or a short interval between the last episode of acute bleeding and capsule endoscopy[28-30], low levels of haemoglobin and high transfusion requirement have been found to be associated with a high diagnostic yield[31,32].

Since capsule endoscopy was introduced in clinical practice 6 years ago, some papers explored also the impact of this new technique on the outcome of patients with obscure GI bleeding. As expected, capsule endoscopy has been found to significantly modify the diagnostic and therapeutic work up immediately after the examination[33], decreasing the number of further examinations and reducing the length of hospital stay[10,34,35]. Nevertheless capsule endoscopy seems also to have a positive impact on long term follow up (mostly evaluated at 12-18 mo after the examination) in about 50%-66%[28,32] of patients and even patients with negative capsule endoscopy have a low probability of experiencing a new bleeding episode (the negative predictive value of capsule endoscopy ranges between 83% and 100%)[34,36].

On the ground of this large amount of published papers capsule endoscopy is now proposed by experts, in patients with obscure GI bleeding, as a third step after a negative bidirectional endoscopy[33], and scientific societies[22,23] define capsule endoscopy as a very valuable tool for investigating obscure gastrointestinal bleeding with the potential capability to improve outcomes.

Recently some Authors evaluated the possible role of capsule endoscopy in the diagnostic work up of patients with isolated iron deficiency anaemia. These studies[37,38], although small, reported that the diagnostic yield of capsule endoscopy in this clinical setting seems quite similar to that reported in patients with obscure GI bleeding (about 50%). These studies confirmed that capsule endoscopy is superior to conventional radiological techniques also in patients with iron deficiency anaemia.

Crohn's disease

We know that Crohn's disease can affect the small bowel: in approximately 45% of Crohn's disease patients the disease involves both the small bowel and the colon and in about 25% the disease is confined to the small bowel, primarily the ileum[39], that can be often difficult to evaluate with endoscopic (retrograde ileoscopy) or radiological methods. For these reasons and because of an increasing number of patients with ulcerative lesions suggesting Crohn's disease[28] has been discovered among subjects undergoing capsule endoscopy for other indications, capsule endoscopy has been also proposed to evaluate the small bowel mucosa of patients with Crohn's disease. However the possible presence of asymptomatic stenoses hampered, at least at the beginning of the experience, the use of this device in patients with a previously established Crohn's disease.

In fact, the first published papers[40,41] on this topic (in 2003) evaluated the diagnostic yield of capsule endoscopy in patients with suspected Crohn's disease (with negative traditional work up, including bidirectional endoscopy and mostly SBFT). The diagnostic yield of capsule endoscopy in this subset of patients (ranging between 33% and 70%)[18,19] has been found, in two independent metanalyses, to be higher when compared with other diagnostic techniques (such as SBFT, SB enteroclysis and retrograde ileoscopy). Marmo *et al*[18] comparing capsule endoscopy with radiological techniques also calculated that the number needed to diagnose (NND) for this subgroup is 2 (95% CI 2-3). Unfortunately the majority of studies aimed at evaluating the role of capsule endoscopy in patients with suspected Crohn's disease included a heterogeneous group of patients, seldom verified over time the final diagnosis by means of other independent diagnostic techniques (i.e. histology), and often used different criteria to classify the lesions identified. A recently published paper[42] tried to overcome possible confounding factors by clearly defining patients with suspected Crohn's disease and by verifying the diagnosis over time. In this paper Girelli *et al*[42] confirmed that capsule endoscopy is an effective tool to diagnose (positive likelihood ratio: 5.8) or to rule out (negative likelihood ratio: 0.08) small bowel Crohn's disease in this particular subset of patients. The authors also pointed out that, in patients with suspected Crohn's disease, assuming a 50% pre-test probability of disease, a positive capsule endoscopy gives a post-test probability of 85%.

The low frequency of capsule retention in patients undergoing capsule endoscopy for suspected Crohn's disease (approximately 1.5%, quite comparable with that reported in patients with obscure GI bleeding)[25]

encouraged the application of this new technique also in patients with established Crohn's disease[43-45]. As expected, in these patients capsule endoscopy showed a high diagnostic yield, significantly superior to that of retrograde ileoscopy and conventional radiological techniques[18,19]. Initial reports comparing capsule endoscopy with CT enteroclysis[46] in patients with established Crohn's disease, although small in size, seem to confirm that capsule endoscopy has a high capability of identifying small inflammatory lesions in the small bowel, and to significantly modify the subsequent management of the patients. However, Golder *et al*[47], using MRI enteroclysis to evaluate the small bowel in patient with Crohn's disease highlighted that, although capsule endoscopy is able to identify a larger number of lesions in the proximal -mid small bowel, in the distal small bowel, which is mostly affected by Crohn's disease, capsule endoscopy and MRI enteroclysis are closely comparable. The authors also pointed out that in these patients MRI enteroclysis identified significant extra intestinal findings in about 30% of cases.

When trying to compare different diagnostic tools for the study of the small bowel in patients with established Crohn's disease, we must keep in mind that capsule endoscopy has been performed exclusively in patients with non stricturing-non penetrating Crohn's disease. In fact all patients in whom a radiological technique showed a stenosis (or a fistula or an abscess) that must be considered as a positive finding of these examinations, were excluded from the comparative studies, leading to a significant and systematic underestimation of the true diagnostic yield of the radiological techniques. Nevertheless, although in the majority of cases, patients with strictures identified with radiological examinations were excluded from capsule endoscopy studies, capsule retention occurred in 5%-13% of cases[25,48]. Capsule retention in patients with established Crohn's disease can be managed, and sometimes partially solved, by giving steroids[49] or using DBE (both for capsule retrieval and stricture dilation)[50,51]. In this subset of patients, capsule endoscopy can be considered as a major complication because it often requires surgical intervention. The development of a dissolvable capsule (see below) may represent, in the near future, the best way to test intestinal patency before capsule endoscopy, in order to avoid capsule retention, especially in patients with Crohn's disease.

Practice guidelines from ESGE[22] suggested that capsule endoscopy, owing to its high diagnostic yield, should have a very important place in the diagnostic work up of patients with known or suspected Crohn's disease, but more large prospective studies are needed to evaluate the specificity of inflammatory lesions, the impact on long term outcome, the clinical significance of the assessment of the extent and severity of small bowel involvement and the risk of capsule retention.

Recent published studies showed also that VCE may have a role in assessing tissue healing after therapy with biologics, relapse after surgical intervention and small bowel evaluation in patients with ulcerative colitis undergoing total colectomy[52].

NSAIDs induced damage

Surprisingly two[13,15] of eight randomized controlled studies published on capsule endoscopy evaluated the role of this technique in assessing small bowel lesions due to NSAIDs consumption. This probably derived from the fact that these widely used drugs can induce small, spotty and superficial mucosal lesions (i.e. mucosal breaks) difficult to identify with other techniques.

Goldstein *et al*[15] clearly demonstrated that NSAIDs (i.e. naproxen), even if associated with proton pump inhibitors (omeprazole), caused more small bowel mucosal lesions than placebo, while Gomez *et al*[13], comparing different NSAIDs, showed that Ibuprofen seems to cause small bowel mucosal damage less frequently than other drugs (dexibuprofen and diclofenac). Another study by Goldstein *et al*[53] comparing COX2-inhibitors with naproxen plus omeprazole showed that among healthy subjects with no endoscopic lesions at baseline, celecoxib was significantly associated with fewer small bowel mucosal breaks than ibuprofen plus omeprazole.

Nevertheless the most important information in this field is the demonstration that small mucosal inflammatory lesions (such as mucosal breaks, small isolated erosion or superficial ulcers) have been detected in about 10%-13% of healthy subjects[15]. Although the clinical implications of these findings remain unclear, the occurrence of these lesions in young healthy subjects, define a new benchmark that must be considered in any further clinical study about capsule endoscopy.

Other indications

As far as celiac disease is concerned two studies[54,55] explored the performance of capsule endoscopy compared with histological evaluation of small bowel biopsies taken during gastroscopy in patients with suspected celiac disease.

Although both studies showed a high agreement between these two techniques (capsule endoscopy sensitivity 85%-87.5%, specificity 90.9%-100%, positive predictive value 96.5%-100% and a negative predictive value of 71.4%-88.9%) the authors underlined that, at present, traditional gastroscopy with duodenal biopsies remains the method of choice to assess mucosal atrophy in patients with suspected celiac disease. However, capsule endoscopy can be a suitable tool in patients, with high clinical suspicion of celiac disease, unable or unwilling to undergo traditional endoscopy.

At the present time, the main obstacle to the extensive use of capsule endoscopy in the diagnosis of celiac disease remains the high costs of the procedure, but also, as highlighted by Biagi *et al*[55], the difficulty in the graduation of mucosal atrophy (see below).

Two studies[57,58], published in 2005 and 2007 respectively, evaluated the role of capsule endoscopy in patients with complicated/refractory celiac disease. In this particular subset of patients capsule endoscopy has been performed to rule out malignant neoplasms [primarily enteropathy associated T-cell lymphoma (EATL)] or other complications (i.e. ulcerative jejunitis). The study of Culliford[57] depicted for the first time the spectrum

of findings (such as scalloping of folds, nodularity and villous atrophy, but also strictures, intussusceptions or submucosal masses), identified by capsule endoscopy in patients with complicated celiac disease, while Daum *et al*[58], demonstrated that capsule endoscopy adds significant clinical information affecting further management mostly in patients with refractory celiac disease type Ⅱ. Nevertheless, both studies included a small number of patients with refractory/complicated celiac disease undergoing a huge number of examinations to exclude strictures; in fact, as previously mentioned for Crohn' s disease, refractory celiac disease can also result in a structuring disease. For these reasons, and mainly because of its capability to take targeted biopsies, double balloon enteroscopy can represent, in this field, a reasonable alternative to capsule endoscopy[59].

Small bowel tumours are still considered, particularly when compared with gastric or colonic neoplasms, a rare disease accounting for 1% to 3 % of all primary gastrointestinal tumours[60], however, since the introduction of capsule endoscopy in clinical practice, some small studies have been published reporting a frequency of small bowel tumours ranging between 6% and 9%[61-65]. These studies, including a series of patients undergoing capsule endoscopy in which this tool was able to identify the presence of small bowel tumours, showed a higher than expected frequency of these tumours. However, two recently presented studies[66,67], published to date only in abstract form, showed a frequency of small bowel tumours ranging between 1.6% and 2.4%. There are no obvious explanations for this discrepancy between studies but the huge number of patients enrolled in the last two studies (more than 6000), the histological confirmation of all reported cases and the substantial concordance with data coming from surgical series, strongly decrease the reliability of earlier data.

All published series about capsule endoscopy in the diagnosis of small bowel tumours underlined that the main clinical indication for capsule endoscopy in these patients is obscure GI bleeding.

In agreement with previously published surgical series, small bowel tumours have been described at capsule endoscopy mostly as polyps (or masses) and stenoses, leading to capsule retention in about 10%[63] to 25%[67] of cases. The most frequent treatment in patients with small bowel tumours is surgical intervention, which, at the same time, allows the retrieval of capsules in case of retention of the device. Therefore, capsule retention in patients with small bowel tumours is considered nowadays as a minor complication.

Capsule endoscopy has also been proposed for the diagnosis and surveillance over time of patients with hereditary polyposis syndromes. The main advantage of this technique in this setting is the capability of this system to inspect the entire small bowel, avoiding radiation exposure and increasing patients' compliance, which is a key point in surveillance programs. Several studies evaluated the possible role of capsule endoscopy in patients with polyposis syndromes[68-70] confirming that, also in this field, capsule endoscopy is more accurate than conventional radiology (SBFT and SB enteroclysis)[71]. Nevertheless the same Authors underlined that the main limitation of this technique, particularly when compared with MRI-enteroclysis, is related to the estimation of size (see below) and location of polyps[68]. At the present time it is suggested that CE should be performed, instead of SBFT, at the time of diagnosis and, as a part of surveillance programs, every 2-3 years, but also as a first diagnostic step in patients with symptoms (i.e. abdominal pain or anaemia)[69,72]. Indeed, keeping in mind the limitations of capsule endoscopy in patients with polyposis syndromes, double balloon enteroscopy can become an important tool, to accurately size and locate lesions, but also to remove polyps identified by capsule endoscopy[73].

The role of CE is less established in patients affected by familial adenomatous polyposis. In fact the quick passage of the capsule through the proximal duodenum can hamper the accurate visualization of the periampullary area. For these reasons, at present, capsule endoscopy is not recommended when the diagnosis of FAP is already established, but may be considered as a part of surveillance for patients with severe duodenal polyposis[69,70]. In a recently published prospective study Wong *et al*[74] compared CE with push enteroscopy and lower endoscopy in 32 patients with FAP. They showed that, in a defined segment of the small bowel, CE diagnosed significantly fewer small-bowel polyps than standard endoscopy, showed only fair agreement with PE in determining polyp counts, and was fairly inaccurate in detecting large polyps and in sizing them.

Abdominal pain, as a possible indication for capsule endoscopy, is still largely debated. Although small bowel tumours have sometimes been identified in patients undergoing capsule endoscopy for unexplained abdominal pain[52], two studies[75,76] evaluating a group of 36 patients with chronic abdominal pain of unknown origin and previous negative diagnostic work-up, found that capsule endoscopy was negative or not clinically relevant in more than 85% of subjects. On the other hand May *et al*[77] clearly demonstrated that when chronic abdominal pain is associated with other signs or symptoms (weight loss > 10% of body weight, inflammation shown by laboratory tests, chronic anemia, or suspected mid-gastrointestinal bleeding) relevant, or potentially relevant, findings are diagnosed by capsule endoscopy in about 60% of cases.

Capsule endoscopy has also been used, with promising results, in other rare clinical conditions such as indeterminate colitis[78,79], small bowel transplantation[80], graft versus host disease[81,82], protein losing entheropathy[83], primitive lymphangectasia[84] (mostly in the pediatric population), Whipple disease[85] and irritable bowel syndrome (with clinical suspicion of celiac disease)[86].

RISKS AND LIMITATIONS

The majority of published papers we mentioned pointed out that the results obtained using capsule endoscopy in clinical practice mainly depend on the revolutionary technical characteristics of this device; however the same technical characteristics can represent, from a certain point of view, limitations of capsule enoscopy. These technical limitations can also explain, in the majority of cases, the clinical limitations of this examination.

Lewis *et al*[87] analyzing a master database, provided

by Given Imaging Ltd (Yoqneam, Israel), found that the global miss rate of capsule endoscopy is about 11% ranging between 0.5% for ulcerative disease and 18.9% for neoplastic disease. Despite the estimated miss rate, capsule endoscopy is significantly lower than that of conventional examinations (global miss rate: 73.3%, miss rate for ulcerative lesions and neoplastic disease: 78.7% and 63.2% respectively) these percentages, in some selected subgroups of patients (i.e. patients with small bowel tumour) are alarming.

Unfortunately there are no conclusive explanations for false negative capsule endoscopies but several factors such as the incompleteness of examination (that can occur in 15%-20% of cases), technical limitations (battery life duration, field of view) and the suboptimal cleanliness of the small bowel (mostly in distal segments) can play a role[88].

At present, although all published papers strongly underlined that small bowel cleanliness is a key point to ensure a complete and accurate examination, and several papers aimed at evaluating factors (dietary restrictions and/or laxatives and/or prokinetic and/or postural tricks) potentially affecting small bowel cleanliness[12,14,16,17,89-93] have been published, there are still no recommendations about small bowel preparation for capsule endoscopy.

This mainly depends on the fact that most studies are published in abstract form, the methodological quality of these studies is rather low, because randomized comparisons are only a small minority, different regimens (with different combinations of drugs) are compared in each study, and an accepted and validated scale to evaluate bowel cleanliness does not exist yet. Four[12,14,16,17] out of 8 controlled randomized studies published on the field of capsule endoscopy are aimed at identifying the best preparation regimen for VCE but, unfortunately, this is only another proof of the relevance of this point.

Despite the lack of any clinical study on this field, all Authors used an overnight fast. An agreement has been reached, basically on the ground of two studies[12,17], about the helpful role of simethicone, administered 20 min before the procedure, in reducing bubbles all along the small bowel, but, the main issue (the presence of liquid stools or fecal debris) which can affect the diagnostic yield of capsule endoscopy, remains to be solved.

In fact, although in 2004[24] the ESGE guidelines, on the ground of the study of Viazis *et al*[16], suggested 2-liters of a poly-ethylen-glycol (PEG) based solution the day before the examination, as small bowel preparation, the updated release of guidelines from the same scientific society (published in 2006)[22] does not recommend any particular schedule of preparation.

The absence of a remote control and of the capability of taking biopsies significantly decrease the specificity of capsule endoscopy findings, since the diagnosis can be based only on the endoscopic appearance. The low specificity of lesions observed at capsule endoscopy is an issue that affects all fields of application of this technique, especially regarding inflammatory lesions (i.e. erosions, ulcers-Figure 4) which can derive from acute and chronic inflammatory bowel diseases[94], ischemic[95], neoplastic[61-67], infectious[96,97] or iatrogenic[98] diseases. As previously mentioned, small and initial inflammatory lesions have also been described in healthy subjects[15].

Another clinical problem strictly dependent on the technical characteristics of the system is the problem of sizing and locating small bowel lesions. This problem, mainly highlighted in studies performed in patients with small bowel hereditary polyposis syndromes[68-70] has important clinical consequences. In fact the size and the location of the lesions are a key point to define, ultimately, the clinical significance of capsule endoscopy findings and to direct further management. In patients with obscure GI bleeding some Authors[99,100] suggested a possible three grade scale (from P0 to P3) to rank capsule endoscopy findings depending on the likelihood of these lesions to explain the reason for referral while, for patients with Crohn's disease two possible scores[43,101] have been proposed but not yet validated. In the field of celiac disease Biagi *et al*[56] clearly demonstrated a large inter- and intra-observer variation in the evaluation of the grade of mucosal atrophy (compared with traditional histology).

In patients with hereditary polyposis syndromes capsule endoscopy tends to overestimate the number of polyps while MRI-enteroclysis seems to be more reliable to correctly estimate the size of polypoid lesions, particularly for polyps of 1-2 centimetres, generally considered clinically relevant[71,74,102].

To improve the capability to estimate the size of polyps Racz *et al*[103] suggested the ingestion, 20' before the procedure, of mesalazine granules as "reference" while Greapler *et al*[104] demonstrated that training with a capsule with a graduated dome might be helpful.

Although awareness of this complication existed at the time of the introduction of this device in clinical practice, the risk of capsule retention at the site of a previously unknown small bowel stricture remains the main complication of capsule endoscopy. This complication seems to be seldom predictable by conventional radiology[105] but the development of a specific dissolvable capsule, even if its safety profile is still under discussion, seems to be a reliable test to screen patients at high risk for capsule retention[107,106-109]. As we know the frequency of capsule retention seems mostly dependent on the clinical indication for capsule endoscopy, ranging between 0% in healthy subjects and 21% in patients with intestinal obstruction[25]. Although capsule retention is the most feared complication of capsule endoscopy, in some selected patients (i.e. patients with small bowel tumor in which capsule retention has been described in 10%-25% of cases) it can be considered as "positive" or minor complication being a sort of "red flag" identifying the presence of the disease. On the contrary, capsule retention must be considered a serious and major complication in patients in whom surgical intervention must be avoided as long as possible (i.e. patients with Crohn's disease, in which capsule retention has been described in about 5%-13% of cases).

Although some case reports described a possible acute obstruction[110] or a possible perforation[111], due to capsule retention, these complications are nowadays considered exceptional. Retained capsules can be retrieved by means of surgical interventions (possibly in a laparoscopic

setting)[112] or by means of enteroscopy (PE or DBE, depending on the site of retention)[113].

Several studies demonstrated the safety of capsule endoscopy in patients with thoracic pace-makers or implanted defibrillators[114-116] and recently a capsule endoscopy has also been performed on a woman in her third trimester of pregnancy because of a life threatening haemorrhage showing a carcinoid tumor[117].

Last but not least, although recently published studies confirmed that this examination is cost-effective in patients with obscure GI bleeding[118], the cost of the procedure can prevent the use of this potentially helpful device in everyday clinical practice. To partially reduce costs of the procedure a possible "two steps" strategy (first step; revision of the video by the nurse and second validation of results by a physician) has been proposed[119,120].

CONCLUSION

Capsule endoscopy, introduced into clinical practice in 2001, revolutionized the study of the small bowel, providing for the first time, a reliable and painless method to evaluate this organ endoscopically. In this paper we critically evaluated the body of evidence produced in these last six years. Unfortunately the majority of published papers are case reports or expert reviews.

Capsule endoscopy has been proven to be significantly superior to conventional radiological techniques (SBFT or SB enteroclysis) for any clinical indication. However studies comparing capsule endoscopy with new imaging techniques (MRI-enteroclysis or CT-enteroclysis) are still few, small and mainly focused on some selected topic (i.e. polyposis syndromes). At the present time capsule endoscopy is recommended as the third examination, after negative bidirectional endoscopy, in patients with obscure GI bleeding. A growing body of evidence suggests also that capsule endoscopy can have a key role in other clinical conditions such as Crohn's disease, celiac disease, small bowel polyposis syndromes or small bowel tumours.

Although awareness of this complication existed at the time of the introduction of this device in clinical practice, the risk of capsule retention at the site of a previously unknown small bowel stricture remains the main complication of capsule endoscopy today. This complication seems to be seldom predictable by means of conventional radiology but the development of a specific dissolvable capsule might, in the near future, provide a safe and reliable test to identify patients at high risk for capsule retention.

Capsule endoscopy still suffers from some technical limitations (there is no remote control, it cannot take biopsies, insufflate air, suck fluids or debris) which can partially explain the clinical limitations/complications of this device (i.e. the difficulty in interpreting inflammatory lesions, in sizing and locating polyps, in grading mucosal atrophy).

The recently developed double balloon enteroscope, owing to its capability to explore a large part of the small bowel and to take targeted biopsies, although invasive and time consuming, can overcome some limitations of capsule endoscopy. At the present time, in the majority of clinical conditions (i.e. obscure GI bleeding), the winning strategy seems to be to couple these two techniques to explore in the most painless, safe and complete way the small bowel (with capsule endoscopy) and to define and treat the lesions identified (with double balloon enteroscopy).

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REVIEW

Telbivudine: A new treatment for chronic hepatitis B

Deepak N Amarapurkar

Deepak N Amarapurkar, Consultant Gastroenterologist, Department of Gastroenterology and Hepatology, Bombay Hospital & Medical Research Centre, Mumbai 400025, India
Correspondence to: Dr. Deepak N Amarapurkar, Department of Gastroenterology and Hepatology, Bombay Hospital & Medical Research Centre Mumbai, D-401.402 Ameya RBI Employees Co-op Hsg Society, Plot No. 947-950, New Prabhadevi Road, Prabhadevi, Mumbai 400025, India. amarapurkar@gmail.com
Telephone: +91-22-4222432 Fax: +91-22-4368623
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Abstract

Three hundred and fifty million people worldwide are estimated to be chronically infected with hepatitis B virus. 15%-40% of these subjects will develop cirrhosis, liver failure or hepatocellular carcinoma during their life. The treatment of chronic hepatitis B has improved dramatically over the last decade merits to the advent of nucleoside/nucleotide analogues and the use of pegylated interferons. Approved drugs for chronic hepatitis B treatment include: standard interferon-alpha 2b, pegylated interferon-alpha 2a, lamivudine, adefovir dipivoxil, and entecavir. Unfortunately, these agents are not effective in all patients and are associated with distinct side effects. Interferons have numerous side effects and nucleoside or nucleotide analogues, which are well tolerated, need to be used for prolonged periods, even indefinitely. However, prolonged treatment with nucleoside or nucleotide analogues is associated with a high rate of resistance. Telbivudine is a novel, orally administered nucleoside analogue for use in the treatment of chronic hepatitis B. In contrast to other nucleoside analogues, Telbivudine has not been associated with inhibition of mammalian DNA polymerase with mitochondrial toxicity. Telbivudine has demonstrated potent activity against hepatitis B with a significantly higher rate of response and superior viral suppression compared with lamivudine, the standard treatment. Telbivudine has been generally well tolerated, with a low adverse effect profile, and at its effective dose, no dose-limiting toxicity has been observed. Telbivudine is one of the most potent antiviral agents for chronic hepatitis B virus and was approved by the FDA in late 2006.

Key words: Telbivudine; Chronic hepatitis B; Hepatitis B virus; Nucleoside analogue; Antiviral agents; Pegylated interferons; Lamivudine; Adefovir dipivoxil; Entecavir

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INTRODUCTION

Hepatitis B virus (HBV) infection is a significant health problem worldwide. Of the 6 billion worldwide populations, an estimated 2 billion have been infected by HBV[1]. It is estimated that 350-400 million people have chronic hepatitis B (CHB) infection[2]. There is clear epidemiologic evidence that chronic HBV infection can result in the development of hepatocellular carcinoma (HCC) and cirrhosis[3,4]. Approximately 15%-40% of HBV carriers develop cirrhosis, liver failure, and HCC; worldwide, more than 50% of primary HCC is related to chronic HBV infection[5]. Each year, 500 000 deaths are expected because of complications related to hepatitis B[6].

EPIDEMIOLOGY IN INDIA

In India nearly 3%-4% of the population is infected by the virus, and chronic hepatitis B constitutes more than 50% of the chronic hepatitis cases in the country[7]. The prevalence ranges from 1.1% to 12.2% with maximum incidence in Madhya Pradesh, Arunachal Pradesh and South India and least in Kashmir and Kerala[8]. There is a peak prevalence after the second decade of life. Most (90%) of these HBV infected subjects are HBeAg negative; the majority (80%) have normal ALT[9]. The prevalence of HBeAg among asymptomatic HBsAg positive persons varies from 9%-20%[9,10].

This, in the context of a large population and absence of a national immunization program would spell off a projected increasing burden of infection and liver disease due to HBV in this country in the years to come. In this perspective, the HBV epidemiology in India becomes relevant not only nationally, but also internationally, because of the possibility that India may soon have the largest HBV infection pool in the world. Economic burden is high and management is affected by cost and availability of diagnostic modalities and patients' awareness and compliance of various treatment options available. Though there have been several guidelines published by various organizations including the Indian association of Study of Liver, the management of HBV infection varies widely in the country. Many physicians in India still find it difficult to make satisfactory management decisions[11].

COST EFFECTIVENESS OF TREATMENT

The main obstacle to treatment in developing countries like India is the expenditure of drug therapy. Cost effective studies have shown savings in countries with intermediate or low endemicity[11]. In India cost of treatment with oral drugs like lamivudine and adefovir range from Rs. 3000 to Rs. 7000/year, a drastic reduction in cost compared to subcutaneous therapy. Additionally the orally used drugs are associated with a good response rate, excellent safety profile and making the overall treatment with oral drugs cost effective. However, the main limitation of these drugs is the emergence of drug-resistant viral strains during the course of treatment.

HBV INFECTION - NATURAL HISTORY

The natural history of CHB is complex and understanding it is important for the selection of patients for treatment. Infected individuals can go through four phases of infection: the immune tolerance phase, the immune reaction or clearance phase (HBeAg-positive, chronic HBV), the inactive carrier (low replication) phase, and the reactivation phase (HBeAg-negative chronic HBV)[1].

In the natural history of HBV infection, the most important event is HBeAg seroconversion characterized by loss of HBeAg and development of antibody to HBeAg (Anti HBe)[12]. The prognosis of chronic HBV infection is dependent upon the amount of inflammation, necrosis and fibrosis in the liver at this point of seroconversion. If significant liver damage is already present at this point, then the prognosis after seroconversion, spontaneous or treatment related is unlikely to be good, despite suppression of viral replication. On the other hand, if the seroconversion has occurred early and is maintained, then the long-term prognosis is excellent. In a subset of persons, this relationship between seroconversion and suppression of viral replication does not hold true. In them, despite anti-HBe positivity, active viral replication persists due to the emergence of mutants in the 'precore' and basal core promoter regions of HBV. This state, characterized by continuing viral replication despite anti HBe positivity has been termed as HBeAg negative CHB[13-15]. Compared with HBeAg-positive CHB, HBeAg negative CHB can follow an aggressive course, requires long or even infinite treatment, and leads to the rapid development of cirrhosis and HCC.

Goals of treatment

The ultimate goal of treatment is the eradication of HBV before it causes irreversible damage including cirrhosis and/or HCC. The eradication of HBV is impossible with currently approved drugs. This is because of extrahepatic reservoirs of HBV, integration of HBV DNA into host DNA, and the presence of covalently closed circular DNA (cccDNA) in the hepatocyte nucleus. Such cccDNA serves as a transcriptional template for HBV replication without the need for reinfection[16,17]. Current antiviral agents have little inhibitory effect on cccDNA, leading to high relapse rates after discontinuation of treatment.

The more realistic goals of therapy are early and prolonged viral suppression, remission of chronic liver disease, a decreased rate of cirrhosis, liver failure, HCC, and reduced morbidity and mortality.

TREATMENT OF CHB

The treatment of CHB has undergone tremendous change and continues to evolve with the advent of potent antiviral agents. The FDA currently approves interferon alpha-2b, pegylated interferon alpha-2a (PEG-IFN-α2a), and four oral agents, adefovir dipivoxil, entecavir, lamivudine and telbivudine as monotherapeutic agents[18].

IFN-α was the first FDA-approved medication for the treatment of CHB. Polyethylene glycol, an inert water soluble molecule when attached to standard interferon to form pegylated interferon decreases the clearance and antigenicity of interferon, thus extending and sustaining its activity *in vivo* allowing for once-weekly administration.

IFN-α and PEG-IFN-α2a have significant side effects, require injection therapy and are less efficacious in patients acquiring CHB during early childhood, for example Asian patients. Furthermore IFN treatment fails to decrease the chance of development of cirrhosis-related complications and HCC[19].

Lamivudine, an oral nucleoside analogue, was the second FDA-approved medication for the treatment of CHB. Viral breakthrough remains a problem with lamivudine with incidence of resistance ranging from 16%-32% after 1 year of therapy to as high as 58% with 2-3 years of therapy[20]. Furthermore, disease progression has been shown to resume following viral breakthrough.

Adefovir dipivoxil, an oral nucleotide analogue, was approved by the FDA in September 2003. Although resistance is less frequent with adefovir compared to lamivudine, this agent may be restricted by nephrotoxicity and a relatively modest potency[18].

Entecavir, a deoxyguanosine nucleoside analog, has recently been licensed by the FDA. Early trials have shown it to be more potent than lamivudine. However, care must be taken when using entecavir in individuals with renal dysfunction[18].

The newest antiviral nucleoside analogues like telbivudine was approved by the FDA based on the results of a Phase Ⅲ clinical trial for the treatment of CHB. The aim of this review article is to introduce and review current information on telbivudine for the treatment of CHB.

TELBIVUDINE

Telbivudine (LdT) is a novel agent for the treatment of CHB. It is an HBV-specific L-nucleoside analogue of thymidine. The chemical name of telbivudine is β-L-2-deoxythymidine (LdT). Telbivudine is an unsubstituted, unmodified β-L-2-nucleoside and the first compound of this series.

Mechanism of action

Telbivudine must be activated by phosphorylation and is efficiently metabolized to 5-triphosphate derivative.

5-triphosphate metabolite of β-L-2-deoxynucleosides interacts with the viral polymerase and inhibits viral replication and results in obligate chain termination of DNA synthesis[21]. This inhibition occurs mainly in the synthesis of the second strand of DNA (DNA-to-DNA transcription) for telbivudine (in contrast to lamivudine which strongly inhibited first strand DNA synthesis; RNA-to-DNA reverse transcription). Since transcription fidelity is higher in DNA-to-DNA synthesis than RNA-to-DNA synthesis, telbivudine treatment may have a slower rate of emergence of drug-resistant virus when compared to lamivudine[21].

Preclinical studies

There is no demonstrable toxic effect on human DNA polymerases α, β and γ by telbivudine after phosphorylation in the experiments using hepatoma cell lines, primary human peripheral blood monocyte cells and human foreskin fibroblasts and other cell types of mammalian origin[21].

In preclinical studies, telbivudine was investigated in rats and monkeys at concentrations substantially greater than the anticipated dose in humans. No significant toxic effects were observed in animal models, suggesting a minimal risk of cumulative, carcinogenic or reproductive toxicity in humans[22].

Pharmacokinetics

A phase Ⅰ/Ⅱ a dose-escalation study in HBV-infected patients showed that telbivudine is rapidly absorbed with the peak concentration reached within approximately 1-3 h. The plasma concentration of telbivudine increased proportionally with increasing doses in the 25-800 mg/d range studied. There were no serious adverse events in all the subjects either receiving telbivudine or placebo[23].

Systemic telbivudine is predominantly cleared unchanged by the kidneys with minimal metabolism and elimination via the hepatic route. Zhou *et al*[24] studied the pharmacokinetic profile of telbivudine administered to patients with moderate to severe renal impairment and hepatic impairment with regards to peak concentration and overall drug exposure [area under the curve (AUC)]. In patients with renal impairment, the AUC was two to three-fold higher in subjects with moderate renal impairment when dosing was normalized to 600 mg/d, suggesting that telbivudine dosage needs to be adjusted in renally impaired patients preferably by reducing the daily dose. Pharmacokinetic profiles were comparable in subjects with normal and impaired hepatic function. The pharmacokinetics are not altered by the food intake[24].

CLINICAL STUDIES

Dose ranging study

The excellent results achieved in the early human PK and safety studies led to the phase Ⅰ/Ⅱ clinical trial in patients with CHB[25]. In this first clinical study of telbivudine, safety, antiviral activity, and pharmacokinetics were assessed in 43 adults with hepatitis Be antigen-positive chronic hepatitis B. This placebo-controlled dose-escalation trial investigated 6 telbivudine daily dosing levels (25, 50, 100, 200, 400, and 800 mg/d); treatment was given for 4 wk, with a 12 wk follow-up. Serum HBV DNA levels were monitored *via* quantitative polymerase chain reaction (PCR). The results indicate that telbivudine was well tolerated at all dosing levels, with no dose-related or treatment-related clinical or laboratory adverse events. Telbivudine plasma pharmacokinetics was dose-proportional within the studied dose range. Marked dose-related antiviral activity was evident, with a maximum of telbivudine doses of 400 mg/d or more. In the 800 mg/d cohort, the mean HBV DNA reduction was 3.75 $\log_{10}$ copies/mL at wk 4, comprising a 99.98% reduction in serum viral load. Correspondingly, post treatment return of viral load was slowest in the high-dose groups[25].

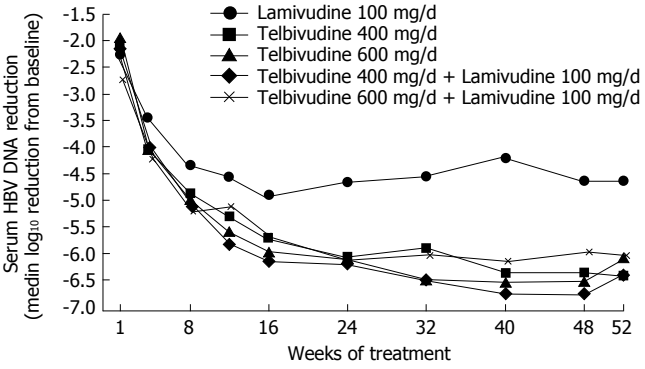

Figure 1 Median reductions in serum hepatitis B virus (HBV) DNA levels at wk 52 (log10 copies/mL) in all treatment groups.

Phase Ⅱb studies

Owing to the encouraging results in the phase Ⅰ/Ⅱ study, a multicenter, international phase Ⅱb trial was initiated. This randomized, double-blind trial evaluated the efficacy and safety of telbivudine 400 or 600 mg/d and telbivudine 400 or 600 mg/d plus lamivudine 100 mg/d (Comb400 and Comb600) compared with lamivudine 100 mg/d in hepatitis Be antigen (HBeAg)-positive adults with compensated chronic hepatitis B[26].

A total of 104 patients were randomized 1:1:1:1:1 among the 5 groups. Median reductions in serum hepatitis B virus (HBV) DNA levels at wk 52 ($\log_{10}$ copies/mL) are shown in Figure 1.

At wk 52, telbivudine monotherapy showed a significantly ($P < 0.05$ for each comparison) greater mean reduction in HBV DNA levels (Figure 1), clearance of polymerase chain reaction-detectable HBV DNA, and normalization of alanine aminotransferase (ALT) levels compared with lamivudine monotherapy, with proportionally greater HBeAg seroconversion and less viral breakthrough (Figure 2). Combination treatment was not better than telbivudine alone. All treatments were well tolerated[26]. This study also examined the prognostic significance of the magnitude of HBV DNA reduction at wk 24 during therapy. For patients with HBV DNA less than 3 log at wk 24 none developed viral breakthrough at wk 52. More importantly 100% of these patients had undetectable HBV DNA by PCR assay at wk 52. In

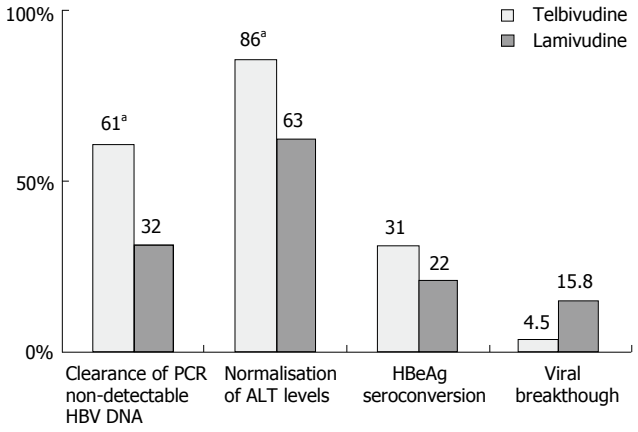

Figure 2 Comparison of telbivudine and lamivudine monotherapy. [a]$P < 0.05$.

addition, these patients had a higher rate of loss of HBeAg and higher chances of ALT normalization at wk 52. These important findings emphasize that treatment should target a rapid and maximal suppression of viral replication[26].

Phase Ⅲ studies

Efficacy *vs* lamivudine: Globe is a phase Ⅲ, double-blinded, randomized, international, multicenter clinical trial designed to compare telbivudine vs lamivudine in over 1369 individuals with CHB over a 2 years period[18].

Individuals entered into the study were screened for HBeAg positivity, and required HBV DNA more than 6 $\log_{10}$ copies/mL by Roche COBAS® Amplicor PCR assay, an ALT greater or equal to 1.3-10 times the upper limit of normal and compensated liver disease. They were stratified for HBeAg status (positive or negative) and ALT value (less or greater than 2.5 times the upper limit of normal). Individuals were randomized to receive 2 years of either: (1) Lamivudine 100 mg qd or (2) Telbivudine 600 mg qd.

The primary end-point of Globe was 'Therapeutic Response', a composite serological end-point comprising suppression of serum HBV DNA to below 5 $\log_{10}$ copies/mL.

Secondary end-points included reduction in serum HBV DNA, normalization of serum ALT, HBeAg loss, seroconversion, and safety[18].

At 1 year[18], a significant reduction in HBV DNA in the telbivudine exposed individuals compared with lamivudine was observed, there being a greater HBV clearance to PCR non-detectable levels in this group also (Table 1). Telbivudine was associated with fewer flares of serum ALT levels when compared to lamivudine. On reviewing histological findings, 65% of HBeAg positive individuals exposed to telbivudine had a significant improvement in histology *vs* 56% of those exposed to lamivudine ($P < 0.01$)[18].

For all clinical and virological efficacy parameters, efficacy at 1 year was proportional to HBV DNA level at wk 24 (Table 2). Achieving HBV DNA < 300 copies/mL at wk 24 with lamivudine or telbivudine was highly predictive of not developing resistance at wk 52. Overall significantly less resistance was seen in the telbivudine arm than the lamivudine arm (2%-3% *vs* 7%-8%). The ability to predict

Table 1 Efficacy at 1 yr in HBeAg positive and HBeAg negative individuals in the Globe study receiving telbivudine or lamivudine

	Telbivudine	Lamivudine	*P* value
HBeAg positive patients			
HBV DNA fall (mean $\log_{10}$)	-6.5	-5.5	< 0.01
HBV DNA non-detectable by PCR (%)	60	40	< 0.01
Treatment failure (%)[1]	5	13	< 0.01
HBeAg negative patients			
HBV DNA fall (mean $\log_{10}$)	-5.2	-4.4	< 0.01
HBV DNA non-detectable by PCR (%)	88	71	< 0.01
Treatment failure (%)[1]	< 1	3	NS

[1]Defined as HBV DNA > 5 $\log_{10}$ copies/mL.

Table 2 Efficacy at 1 yr against virological suppression at 24 wk in HBeAg positive and negative individuals in the Globe study

	Virological suppression at wk 24 (%)			
	< 300 copies/mL	300-1000 copies/mL	> 1000-10000 copies/mL	> 10000 copies/mL
HBeAg positive patients (wk 52)				
HBV DNA (< 300 copies/mL)	90	70	30	5
ALT normalization	90	89	80	54
Viral breakthrough	1	3	8	11
HBeAg negative patients (wk 52)				
HBV DNA (< 300 copies/mL)	93	66	38	10
ALT normalization	83	74	63	36
Viral breakthrough	0	9	18	32

subsequent outcomes at 24 wk enables clinicians to estimate a response and plan future therapeutic interventions.

The 2 years results of this study[27] were presented recently at the AASLD annual meeting at Boston. At 2 years telbivudine showed significantly greater therapeutic response, a greater reduction in HBV DNA from baseline and greater HBV DNA clearance to PCR negative. Telbivudine showed significantly less primary and secondary failure, breakthrough and resistance. Clinical adverse event profiles were similar between the two treatment groups (Table 3). Both study drugs were generally well-tolerated, with similar patterns of clinical adverse events. Clinical and virological efficacy at 2 years was also linked to magnitude of HBV suppression at wk 24 (Table 4)[28].

Another phase Ⅲ trial compared telbivudine with lamivudine in Chinese individuals with CHB[29], 87% of whom were HBeAg positive. At 52 wk, 70% of telbivudine exposed individuals had undetectable HBV DNA levels (defined as < 300 copies/mL by PCR) compared with only 43% of lamivudine treated individuals ($P < 0.001$). Telbivudine was superior to lamivudine in normalizing ALT levels (89% *vs* 76%, respectively, $P < 0.005$). In telbivudine exposed individuals who were HBeAg positive, on entering the study, 25% had seroconverted at 52 wk, compared with 18% of those treated with lamivudine. Both Telbivudine and lamivudine were equally well tolerated.

Table 3 Efficacy at 2 yr in HBeAg positive and HBeAg negative individuals in the Globe study receiving Telbivudine or lamivudine

	Telbivudine	Lamivudine	*P* value
HBeAg positive patients			
HBV DNA fall (mean $\log_{10}$)	-5.7	-4.4	< 0.05
HBV DNA non-detectable by PCR (%)	54	38	< 0.05
Treatment failure (%)[1]	4	12.3	< 0.05
HBeAg negative patients			
HBV DNA fall (mean $\log_{10}$)	-5	-4.2	< 0.05
HBV DNA non-detectable by PCR (%)	79	53	< 0.05
Treatment failure (%)[1]	0	3	< 0.05

[1]Defined as HBV DNA > 5 log10 copies/mL.

Efficacy *vs* adefovir: A third major phase Ⅲ study has compared the use of telbivudine and adefovir in HBeAg positive individuals with CHB for 24 wk[30]. At the end of the study, a significantly greater HBV DNA reduction was seen in those individuals exposed to telbivudine (6.37 *vs* 5.11 $\log_{10}$ copies/mL; $P < 0.01$). In individuals exposed to adefovir, 42% failed to reach a HBV DNA below 5 $\log_{10}$ copies/mL, compared with 5% in the telbivudine arm ($P < 0.01$). There was no significant difference in HBeAg loss or normalization of ALT levels between the two arms. There was no difference in adverse events between the two arms.

Resistance: Of the 1367 individuals included in the Globe ITT analysis, 81 patients experienced viral breakthrough[18]. Among these, genotypic resistance was confirmed in 69:17 telbivudine-treated patients and 52 lamivudine recipients. Following sequencing, all resistance was associated with M204 variants in the YMDD motif of the genome. Individuals failing lamivudine had acquired either M204I, M204V or a mixed picture of M204M/I/V. In those exposed to telbivudine, M204I was the only mutation detected in 16 of 17 telbivudine patients with resistance; the other patient carried a mixture of M204M/I/V.

The M204V lamivudine resistant mutation was associated with the 180M compensatory mutation, thus forming a double mutant, whereas the M204I telbivudine mutation was not. These results imply that telbivudine may suppress the emergence of fully resistant HBV *via* the M204V pathway that is dominant with lamivudine.

FUTURE TRENDS IN THE MANAGEMENT OF CHRONIC HEPATITIS B

Emerging data suggest that the magnitude of reduction in serum HBV DNA levels that are achieved early in the course of therapy with nucleos(t)ides predicts the likelihood of subsequent efficacy outcomes. Data from the GLOBE study suggest that patients who have rapid and profound viral response to treatment (serum HBV DNA < 300 copies/mL at 24 wk) tend to have a higher probability of maintaining the response and a lower probability of resistance over time. Conversely, patients who have a lower initial viral response tend to have a higher probability of low rates of response and resistance over time.

Table 4 Efficacy at 2 yr against virological suppression (PCR negative) at 24 wk in HBeAg positive and negative individuals in the Globe study

	Yr 2 outcome	Probability (%)
HBeAg positive	HBeAg seroconversion	45
	ALT normalization	79
	HBV DNA non-detectable by PCR	77
HBeAg negative	ALT normalization	77
	HBV DNA non-detectable by PCR	74

Understanding the factors that predict poor response and resistance can potentially permit treatment modification to optimize response to treatment. Therefore, the response to a single agent after 24 wk of therapy may be used effectively by the clinician to determine whether the patient is likely to benefit from continued monotherapy or if the addition of another agent may offer a better probability for improved long term outcomes.

A clinical trial to assess this approach with telbivudine will be conducted in India. The trial design will involve assessment of response, based on serum HBV DNA levels, at specific early time points starting at 24 wk. Based on the degree of viral suppression at these time points, telbivudine may be continued or another agent could be added.

CONCLUSION

Owing to its good safety profile and high antiviral potency, telbivudine is one of the most promising drugs for the treatment of CHB. Telbivudine is one of the new β-L-nucleoside analogues with potent antiviral activity against HBV. Telbivudine is an obligate chain terminator, incorporating into HBV DNA, and exerts a preferential effect on second strand DNA synthesis. The promising results of the early *in vitro* and animal studies paved the way for phase Ⅰ/Ⅱ human clinical trials. Phase ⅡB human clinical studies demonstrated superior antiviral efficacy of telbivudine, significantly better ALT normalization and better HBeAg loss as compared with lamivudine. Further large international multicenter phase Ⅲ studies have confirmed these results with telbivudine in comparison not only to lamivudine but also to adefovir.

Overall, telbivudine appears to be efficacious, easy to take with a good safety profile, proving to be a valuable therapeutic option in the management of hepatitis B.

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TOPIC HIGHLIGHT

Jesús K Yamamoto-Furusho, *Series Editor*

Basic and clinical aspects of osteoporosis in inflammatory bowel disease

Lorena Rodríguez-Bores, Josué Barahona-Garrido, Jesús K Yamamoto-Furusho

Lorena Rodríguez-Bores, Josué Barahona Garrido, Jesús K Yamamoto-Furusho, IBD Clinic, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Vasco de Quiroga 15 colonia Sección XVI, Tlalpan, CP 14000, México
Correspondence to: Jesús K Yamamoto-Furusho, MD, PhD, Head of IBD Clinic, Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Vasco de Quiroga 15 colonia Sección XVI, Tlalpan, CP 14000, México. kazuofurusho@hotmail.com
Telephone: +52-55-55733418-2705 Fax: +52-55-56550942
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Abstract

Low bone mineral density and the increased risk of fracture in gastrointestinal diseases have a multifactorial pathogenesis. Inflammatory bowel disease (IBD) has been associated with an increased risk of osteoporosis and osteopenia and epidemiologic studies have reported an increased prevalence of low bone mass in patients with IBD. Certainly, genetics play an important role, along with other factors such as systemic inflammation, malnutrition, hypogonadism, glucocorticoid therapy in IBD and other lifestyle factors. At a molecular level the proinflammatory cytokines that contribute to the intestinal immune response in IBD are known to enhance bone resorption. There are genes influencing osteoblast function and it is likely that LRP5 may be involved in the skeletal development. Also the identification of vitamin D receptors (VDRs) and some of its polymorphisms have led to consider the possible relationships between them and some autoimmune diseases and may be involved in the pathogenesis through the exertion of its immunomodulatory effects during inflammation. Trying to explain the physiopathology we have found that there is increasing evidence for the integration between systemic inflammation and bone loss likely mediated via receptor for activated nuclear factor kappa-B (RANK), RANK-ligand, and osteoprotegerin, proteins that can affect both osteoclastogenesis and T-cell activation. Although glucocorticoids can reduce mucosal and systemic inflammation, they have intrinsic qualities that negatively impact on bone mass. It is still controversial if all IBD patients should be screened, especially in patients with preexisting risk factors for bone disease. Available methods to measure BMD include single energy x-ray absorptiometry, DXA, quantitative computed tomography (QCT), radiographic absorptiometry, and ultrasound. DXA is the establish method to determine BMD, and routinely is measured in the hip and the lumbar spine. There are several treatments options that have proven their effectiveness, while new emergent therapies such as calcitonin and teriparatide among others remain to be assessed.

Key words: Inflammatory bowel disease; Osteoporosis

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INTRODUCTION

Patients with inflammatory bowel disease (IBD) are at increased risk of developing disorder in bone and mineral metabolism because of several factors, including the genetic influence, cytokine-mediated nature of the inflammatory bowel disease, the intestinal malabsorption resulting from disease activity or from extensive intestinal resection and the use of glucocorticoids to control disease activity. Apparently these disturbances may also be seen since childhood, and environmental factors such as malnutrition, immobilization, low body mass index (BMI), smoking and hypogonadism may also play a contributing role in the pathogenesis of bone loss. In IBD several studies demonstrate a negative correlation between bone mineral density (BMD) and glucocorticoid use, though there is evidence that may support the opposite. In order to answer the questions about the pathogenesis, we first have to determine the factors that are involved in this extraintestinal complication. The aim of this paper is to review the basic and molecular aspects with the clinical and therapeutic features and have an overview about the trends of the bone disease related to IBD.

EPIDEMIOLOGY

Bone mineral density is decreased in a proportion of subjects with IBD as shown by epidemiological studies. The current understanding about IBD and BMD is that

the overall risk of fracture may be slightly increased in IBD patients. IBD has been associated with an increased risk of osteoporosis and osteopenia and epidemiologic studies have reported an increased prevalence of low bone mass in patients with IBD. The prevalence rates from 2% to 30% for osteoporosis (OP), from 40% to 50% for osteopenia[1] and the overall prevalence of low bone mineral density is estimated in 15%. A population-based study compared IBD patients with the general population and reported similar increases in the fracture risk between Crohn's disease (CD) and Ulcerative colitis (UC)[2] and in comparison to control patients, similar to what other population-based studies have reported[3,4]. Some series have reported that in newly diagnosed IBD patients a reduced BMD has been found and this prevalence is slightly higher in patients with CD[3,5] whereas approximately 15% of patients with CD have osteoporosis[6]. There is contrasting data from a Danish case-control study where an increase in the risk of fracture among women with CD was seen, but not men with CD or patients with UC[7,8], also another study reported that the overall fracture rate in UC was similar to that of control subjects[9]. In regard to age and gender as risk factors, elderly have the highest risk of fracturing and this increased risk is evident across all age groups[3]. Some case control studies have demonstrated that gender, age, and body weight are the major determinants of bone mineral density in patients with CD. As in healthy individuals, the combined effect of these factors account for up to 50% of the variability in bone mineral density[10]. Male sex and increasing age were considered risk factors in predicting those with osteoporosis although most series report no significant difference between the genders.

Longitudinal studies show that the BMD changes are not excessive[11,12] and there is no exclusive pattern of low BMD that involves spine of the hip. The risk of hip fracture is increased by 86% in patients with CD and by 40% in patients with UC[2]. However the hip has been reported more frequently affected than the spine[13,14]. In a study of Stockbrugger *et al* significant number of fractures in IBD patients as in the general osteoporotic are asymptomatic, about 14.2% of the fractures seem to be underreported[15], though it is important to mention that osteoporosis occurrence is often underestimated[15].

GENETICS

Low bone mineral density and the increased risk of fracture in all gastrointestinal diseases including IBD have a multifactorial pathogenesis. There are a number of factors that can lead to enhanced bone loss, these also include genetic factors.

LRP5

Because of the central role of osteoblasts in bone formation, it is easy to think there are genes influencing osteoblast function and it is likely that LRP5 may be involved in the skeletal development. The protein encoded by *LRP5* is a member of the low-density lipoprotein-receptor (*LDLR*) gene superfamily[16] and is closely related to *LRP6*[17]. *LRP5* is transcribed in human bone tissue as well as in numerous other tissues. There is convincing findings that deleterious (loss of function) mutations in *LRP5* result in loss of function and cause bone defects such as the ones seen in pseudoglioma syndrome further supporting the critical role of this gene in skeletal integrity[18]. There is some data about the identification in normal healthy individuals of a gain of function mutation in the LDL receptor-related protein 5 (LPR5) gene resulting from a autosomal dominant high bone mass trait[19] and this gain of function mutation described in LRP5 produces increased bone mass with no adverse effect on skeletal structure, contrasting the loss of function mutation that maps to the same genomic region that contains LRP5 causes the osteoporosis pseudoglioma syndrome[20]. Polymorphisms rs491347 rs1784235 could be important to human osteoporosis phenotypes and may be considered as possible susceptibility factors for osteoporosis and fractures in humans[21]. A Japanese study found that the A1330 V polymorphism may contribute to osteoporosis susceptibility[22] and also was associated with reduced BMC and BMD values in healthy young Finnish men, providing evidence for the crucial role of LRP5 in peak bone mass acquisition[23].

VDR (Vitamin D receptor gene)

The identification of vitamin D receptors (VDRs) in peripheral blood mononuclear cells sparked the early interest in vitamin D as an immune system regulator[24]. Vitamin D deficiency has been linked to several different diseases, including the immune system-mediated OP such as IBD. The association of VDR gene BsmI polymorphism with OP has been studied by several investigators[24-28]. In addition, TaqI, FokI and ApaI polymorphisms of the VDR gene have also been described[25]. Regarding OP, most data concern to the BsmI polymorphism of the vitamin D receptor (VDR) gene.

Candidate genes

There are other candidate genes that seem involved with bone loss. Estrogen receptor alpha (ER alpha) play an important role in increasing BMD *via* mechanical strain and muscle mass[29]. The results of studies regarding the association between some common polymorphisms of the aromatase gene and bone mineral density and the risk of osteoporotic fractures are recognized[30]. Thus, aromatase is also an attractive osteoporosis candidate gene. The gene encoding TGFβ1 is a strong functional candidate for genetic susceptibility to osteoporosis. Several polymorphisms have been identified in TGFβ1, and previous work has suggested that allelic variants of TGFβ1 may regulate BMD and susceptibility to osteoporotic fracture[31]. During the last years, about 170 candidate genes have been published. There have been (e.g., VDR, ER-α, and COL1A1), as well as novel genes recently discovered to be important in bone and mineral metabolism. The newly studied genes include a big list CYP17 (17-hydroxylase)[32], CYP1B1 (cytochrome P450)[33], DBP (vitamin D-binding protein)[34], GH1 (growth hormone 1)[35], GnRH (gonadotropin-releasing

hormone 1[35]), IGF-Ⅱ (insulin-like growth factor Ⅱ)[37], LEPR (leptin receptor)[38], LRP5 (low-density lipoprotein receptor-related protein 5)[39], BMP2 (bone morphogenetic protein 2)[40], CCR2 (chemokine)[41], CLCN7 (chloride channel 7)[42], COMT (catechol-O-methyltransferase)[43], CTSK (cathepsin K)[44], DRD4 (dopamine receptor D4)[45], I-TRAF (TRAF family member-associated NF-κB activator)[46], LCT (lactase)[47], MIF (macrophage migration inhibitory factor)[48], MMP-1 (matrix metalloproteinase 1)[49], among many others, but their relationship with inflammation as a possible mechanism of osteoporosis still is not clear and the interaction with IBD bone disease has not been elucidated. The mechanisms involved and the potential usefulness of those genetic data in the prevention and management of osteoporosis need further investigation, also to determine the direct relation with IBD.

PATHOPHYSIOLOGY

Inflammation has now moved to the center of the physiopathologic mechanisms involved in the process of bone loss in IBD, there has been a considerable increase in knowledge surrounding the genetic determinants of osteoporosis. As well as genetic markers are potentially helpful in identifying high risk patients, the genetic variations of cytokines plays a key role in the regulation of the inflammatory response. Several studies are focused trying to identify genetic risk factors for rapid bone loss in IBD patients as a model of disease and inflammation-associated bone loss. Evidence accumulated in the past years support that interleukin 6 (IL-6) is a pathogenic factor in osteoporosis that results from the loss of either male or female sex steroids and have implicated IL-6 in the physiopathology of several other diseases caused by increased osteoclastic bone resorption including diseases such as Rheumatoid arthritis[50]. Genetic variations in the IL-6 and interleukin 1 receptor antagonist (IL-1ra) gene identify IBD patients at risk for increased bone loss. Allele status of the IL-1ra, IL-6, heat shock protein 70-2 and 70-hom (hsp 70-2, hsp hom) gene has been typed and correlated with clinical course of IBD and extent of bone loss[51]. These variations are independent determinants of bone loss in the setting of IBD, and have been identified as independent predictors of bone loss in the setting of postmenopausal osteoporosis, suggesting that IL-6 and IL-1ra determine the response of bone to different stressors such as the hypoestrogenic state or systemic inflammation[52,53]. Apparently, estrogen loss results in increased production of IL-6 by *ex vivo* bone marrow cell cultures and increased production of IL-6 follows the withdrawal of estradiol from primary culture[54,55]. It seems that IL-6 is responsible for increased bone resorption after loss of sex steroids and that gonadectomy prevents the increase in osteoclastogenesis in bone marrow and the increase in the number of osteoclasts in sections of trabecular bone[56]. The cytokines IL-1ra and IL-6 also have a central role in the paracrine stimulation of osteoclast development and regulation of the process of bone resorption[50,55]. Increasing evidence suggests that IL-6 type cytokines also promote the development of osteoblasts[50]. It has been observed that the carriage of the A2 allele of the IL-1ra gene is associated with reduced bone loss[52].

The interleukine-2 (IL-2) deficient mouse model of colitis is known to develop both osteopenia and colitis. Osteopenia was not evident in IL-2 deficient mouse cross-bred to be T-cell deficient, and osteopenia could be induced in T-cell-deficient mice by adoptive transfer of T cells from IL-2 deficient mice[57]. These data suggest that activated T cells are critical for mediating the osteopenia.

OPG-RANK-RANKL system

The receptor activator of nuclear factor κB ligand (RANKL) osteoprotegerin (OPG) system represents a potential link between inflammation and bone homeostasis and also an example of inflammation-mediated osteopenia such as IBD-associated osteopenia. The balance between RANKL and OPG (the soluble decoy receptor preventing ligation of RANKL) is of major importance to the regulation of osteoclastogenesis. The interaction of RANK on the surface of osteoclasts with its ligand RANKL induces osteoclastogenesis and conversely the interaction with the osteoblast derived soluble decoy receptor, osteoprotegerin (OPG)[58] blocks RANK-RANKL interaction inhibiting osteoclasts formation. Whether compounds stimulate RANK ligand or OPG will affect whether they induce or inhibit osteoclastogenesis. Pro-inflammatory cytokines induce RANKL and promote bone resorption with consecutive bone loss. Activated T cells can directly trigger osteoclastogenesis through RANKL leading to bone loss while OPG can block those effects[59-61]. Increased OPG levels may represent a continuing homeostatic response, attempting to reverse established osteopenia and RANKL driven osteoclastogenesis, thus maintaining normal bone mass. Inflammation seems to play an important role in the regulation of the OPG-RANK-RANKL system. To correlate it with chronic inflammatory states comparable to IBD, there have been some reports that show a direct correlation between serum OPG and erythrocyte sedimentation rate and a score of disease activity in patients with rheumatoid arthritis[62]. Soluble RANKL as well as OPG levels are elevated in rheumatoid arthritis, while high OPG and decreased RANKL levels have been reported in primary biliary cirrhosis[63,64]. Some of the osteoclastogenic factors released from the IBD mucosa (for example IL-1, IL-6 and TNFα) are thought to function indirectly via specific receptors on stromal osteoblastic cells to enhance RANKL expression[60,65,66]. Data suggests that OPG may be a protective host response that partially offsets the adverse skeletal effect created by the inflammation state. Moshen *et al*[67] described the alterations in the RANKL/OPG system in IBD and its relationship to decreased BMD. It has been demonstrated increased plasma levels of OPG as well as increased release from the inflamed colon in IBD, suggesting the macrophages and dendritic cells as colonic source of OPG in IBD. Apparently, no correlation was evident between corticosteroid and serum OPG[63] contrasting partially with other findings.

Corticosteroids

The controversial participation of glucocorticoid (GC) therapy in the pathogenesis of bone loss in IBD still has gaps to be fulfilled. It seems that there is an important relationship between dosage, duration and pattern of GC therapy and these factors are related to the incidence of pathological fractures[68]. Some studies indicate that fractures are present in 30%-50% of patients on GC therapy for chronic diseases[69] and several studies have demonstrated that dosage is associated with BMD[51,70-73]. On the other hand, several studies have reported the opposite[8,13].

The epidemiological data on fracture risk and bone loss in GC therapy do not distinguish the effects of drug and the effects of the underlying disease. It is known, for example, in rheumatoid arthritis, the risk of fracture is increased even in the absence of GC exposure, also it has been observed that osteoporosis is rapidly developed in recently diagnosed Crohn's disease without any effect of corticosteroids in the follow up. One study showed that the prevalence of osteoporosis in pediatric patients with IBD is approximately the same as in adult patients, showing that osteoporosis was already present before steroid treatment[74]. Contrasting data from other studies show that the extent of bone loss was no correlated to clinical severity of disease or application of corticosteroids[75-77]. The participation of GC in the pathophisiology of bone loss is complex. GCs influence the production and action of hormones that regulate bone and calcium metabolism and also have direct effects of GCs on bone. GCs increase the expression of receptor activator of nuclear factor κB ligand (RANK-L) and decrease the expression of its soluble decoy receptor osteoprotegerin (OPG) in stromal and osteoblastic cells[78] and also enhance the expression of macrophage colony-stimulating factor (M-CSF), which in the presence of RANK-L induces osteoclastogene sis[78-80]. GCs have direct effects on osteoclasts also by suppressing the expression of an autocrine cytokine, such as interferon I , that normally exerts inhibitory effects on osteoclastogenesis[80]. Also they inhibit the function of mature osteoblasts and suppress the synthesis of insulin-like growth factor- I , an agent that enhances bone formation[78,79].

The wingless-type (Wnt) signaling has emerged as a novel, key pathway for promoting osteoblastogenesis. The Wnt signal transduction comprises three intracellular pathways: the canonical pathway, the Wnt/planar-cell-polarity (PCP) pathway, and the Wnt/Ca^{2+} pathway[81,82]. Wnt signals are extracellularly regulated by several secreted antagonists including secreted frizzled-related protein (sFRP), Cerberus, Wnt inhibitory factor-1 (WIF-1), and dickkopf (Dkk)[83]. Some studies strongly suggest that the canonical pathway plays a central role in promoting bone formation[84-86]. Some groups have reported that glucocorticoid enhances the expression of dickkopf-1 (Dkk-1) in cultured human osteoblasts[87] by suppressesing the canonical Wnt signal[88].

DIAGNOSIS

Diagnosis of osteoporosis in IBD patients

Due to the low absolute risk of fracture remains controversial if all IBD patients should be screened, but it is suggested for avoiding the complications of osteoporosis, especially in patients with a preexisting bone disease, older than 65, and with risk factors for low bone mass as long-term steroid therapy (prednisone 5 mg daily for 6 mo or more)[88-91].

Both, the American College of Gastroenterology (ACG) and American Gastroenterological Association (AGA) issued position papers to offer guidance to the practicing clinician in the diagnosis and management of bone loss in IBD. These position papers recommended the selective screening of IBD patients with dual energy x-ray absorptiometry (DXA) scanning, and the criteria for DXA screening included: postmenopausal state , ongoing corticosteroid treatment, cumulative prior use of corticosteroids exceeding 3 mo, history of low trauma fractures, and age over 60. These criteria led to the detection of osteopenia or osteoporosis and initiation of specific therapies in the majority of patients[92].

Available methods to measure BMD include single energy x-ray absorptiometry, DXA, quantitative computed tomography (QCT), radiographic absorptiometry, and ultrasound. DXA is the establish method to determine BMD, and routinely is measured in the hip and the lumbar spine[93].

The T score was proposed by the World Health Organization (WHO) as the strongest determinant of fracture risk. T score is defined as the number of standard deviations (SD) by which a given BMD measurement exceeds or falls below the normal mean BMD of healthy 30-year-old individuals (peak bone mass). A BMD that is up to 1 SD below the peak bone mass is considered normal; between 1 to 2.49 SD below peak BMD is considered as osteopenic and to have mild to moderate bone deficiency; and $\geqslant$ 2.5 SD below the peak BMD are labeled osteoporotic and with marked bone deficiency. Individuals who have a fracture as a result of bone fragility are considered to have severe osteoporosis[93]. The z score is useful too, and is defined as the number of SDs by which a given BMD measurements exceeds or falls below the mean BMD of healthy individuals of the same age group. For the International Society for Clinical Densitometry (ISCD), z scores are preferred, and the WHO classification should not be applied in women before menopause and in men younger than 50[94].

TREATMENT OPTIONS

Calcium and vitamin D

It is known that calcium and vitamin D are essential in the metabolism of bone and so multiple trials have studied their benefit as treatment of osteoporosis. The use of calcium or/and vitamin D or its analogues have shown, in 2 meta-analysis, 1 Cochrane Review, and in a large placebo-controlled study, to have a small benefit in BMD and a controversial age-dependant trend, and not totally clear reduction of bone fractures, specially of the spine, in postmenopausal women[95-98]. In a randomized, placebo-controlled trial in glucocorticoid-using patients with IBD, the intake of vitamin D 250 IU and calcium 1000 mg/d had no significant benefit in bone density at 1 year of follow-up[99]. So, as described in a recent

consensus report, the supplementation with calcium and vitamin D is accepted as a cost-effective medication, and essential but insufficient, in the prevention and treatment of osteoporosis. The dosage that showed best is calcium 1200 mg/d and vitamin D 800 IU/d. The maximum benefit of calcium and vitamin D will generally be derived from combination therapy with an antiresorptive agent[100].

Bisphosphonates

The group of this antiresorptive analogue of pyrophosphate includes etidronate, pamidronate, tiludronate, alendronate, risedronate, and ibandonate.

Both, alendronate and risedronate, have shown to be effective in increasing BMD and reducing fractures in spine, hip and wrist for the treatment of osteoporosis in postmenopausal women. In a systematic review, meta-analysis and double blind and randomized study, they reduce vertebral fractures by 30% to 50%, with superiority for 70 mg once-weekly alendronate than daily 5 mg or once-weekly 35 mg of risedronate, and with similar tolerability profiles, at 1 or 2 years[101-105].

For the prevention and treatment of glucocorticoid-induced osteoporosis, in a randomized, double-blind, placebo-controlled, multicenter study, in patients receiving a minimum of 7.5 mg prednisone or its equivalent for diverse pathologies, all receiving 800-1000 mg elemental calcium and 250-500 IU of vitamin D, alendronate at a dosage of 5 or 10 mg/d significantly increased bone density compared to placebo at 1 year and reduced the incidence of bone fractures too, at 2 years[106,107].

In patients with moderate to high doses of corticoid therapy, a significant increase of BMD and a reduction of 70% in vertebral fracture risk was observed with risedronate 5 mg/d compared with the placebo group ($P = 0.01$). Risedronate was efficacious, irrespective of underlying disease and duration of corticosteroid therapy, and had a favorable safety profile, with a similar incidence of upper gastrointestinal adverse events to placebo[108,109].

Etidronate have shown to be superior to placebo for increasing BMD in lumbar spine and femoral neck, and reducing incidence of vertebral fractures with no effect in non-vertebral fractures in postmenopausal women[110].

A meta-analysis reported that intermittent cyclical etidronate (400 mg/d for 14 d, followed by 500 mg calcium daily for 76 d) in corticoid treated patients was effective in preventing bone loss, increasing bone mass but with no statistical significance on reduction of fractures[111].

Other bisphosphonate approved for the treatment of osteoporosis in postmenopausal women is the ibandonate in oral dosage of 2.5 mg/d, or intravenous dosage of 2 mg every 2 mo, or 3 mg every 3 mo, had shown to be better than placebo, increasing BMD and reducing bone fractures, with superiority of intravenous regimens[112].

For corticoid-induced osteoporosis, in an open-label, single-center, parallel-group, controlled study, participants received 500 mg/d calcium plus either 3-montly intravenous injections of 2 mg ibandronate or oral 1 mg/d alfacalcidol for 3 years, showing that the increase in BMD was much greater and the fractures were lower in the ibandronate than those in alfacalcidol group[113].

For the treatment of osteoporosis in IBD, bisphosphonates have been evaluated in few studies. In a 12-mo double-blind, randomized, placebo-controlled study of 10-mg daily dose of alendronate, that include 32 patients with CD in remission and without glucocorticoid treatment the BMD of the lumbar spine increased 4.6% ± 1.2% versus a decrease of 0.9% ± 1.0% in the placebo group ($P < 0.01$). BMD of the hip increased 3.3% ± 1.5% *vs* an increase of 0.7% ± 1.1% in the placebo group ($P < 0.08$)[114].

In 31 patients with CD and 30 with UC, in a double-blind placebo-controlled study, all taking 600 mg daily of calcium, after 1 year in the risedronate group the BMD of the spine and hip significantly increase in 2% and 1.9%, respectively[115]. After one year of monthly infusions of 30 mg iv pamidronate plus 500 mg calcium with 400 IU vitamin D in patients with CD, the BMD increased 2.6% (95% CI: 1.4-3.0) at the spine and 1.6% (95% CI: 0.6-2.5) at the hip versus 1.6% (95% CI: 0.1-3.2) at the spine and 0.9% (95% CI: 0.4-2.1) at the hip in the group with vitamin D and calcium supplements[116]. Stokker PC *et al*[117] reported a significant improve in T scores of lumbar spine and hip in 49 patients with IBD that received 30 mg iv pamidronate every 3 mo, plus 1000 mg of calcium and 400 IU of vitamin D daily.

Estrogens

Estrogens alone or with progestin stop progression of bone loss in postmenopausal women, increasing the BMD and reducing the incidence of spine and hip fractures by 34%[118]. Good response in preventing bone loss in patients under glucocorticoid treatment has been observed but the effect on prevention of bone fractures remains unclear, estrogens are not recommended for this purpose[119,120].

Raloxifene, a selective estrogen receptor modulator was approved for the prevention and treatment of postmenopausal spinal osteoporosis. In a meta-analysis of 7 clinical studies, raloxifene reduced the risk of vertebral fractures by 40% with a dose of 60 mg/d[121]. No studies with raloxifene have done yet in IBD patients.

EMERGENT THERAPIES

Calcitonin

Calcitonin intranasal spray, at doses of 200 IU/d plus 1000 mg calcium and 400 IU vitamin D, has been reported to reduce the risk of spine fractures by 33% in a 5-year follow-up time in postmenopausal women[122].

The efficacy of calcitonin for fracture prevention in steroid-induced osteoporosis remains to be established[123,124]. No studies have done for IBD-associated osteoporosis.

Teriparatide

The genetically engineered fragment of human parathyroid hormone, Teriparatide, stimulates new bone formation, leading to increased BMD. Teriparatide, at 20 and 40 micrograms daily subcutaneous injection, reduced the risk of vertebral and non-vertebral fractures in postmenopausal women[125]. It's also approved for FDA to increase bone mass in men with primary or hypogonadal osteoporosis[126].

The efficacy of teriparatide in preventing of treating glucocorticoid-induced or IBD-associated osteoporosis remains to be assessed. Hodsman AB[127] suggests that should be considered as treatment for patients with established glucocorticiod-induced osteoporosis who require long-term steroid treatment.

CONCLUSION

IBD has been associated with an increased risk of osteoporosis and osteopenia and epidemiologic studies have reported an increased prevalence of low bone mass in patients with IBD. While genetics play important role, there are other factors in the pathogenesis that play an important interaction and together with environmental influence lead to the an intriguing multifactorial pathogenesis that still has gaps to be fulfilled. Through the knowledge and understanding of basic aspects of bone disease in an autoimmune gastrointestinal scenario we can find leads to a better clinical performance and to bear new diagnostic techniques and breakthrough therapies for a better outcome in IBD patients.

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GASTRIC CANCER

Re-expression of methylation-induced tumor suppressor gene silencing is associated with the state of histone modification in gastric cancer cell lines

Chun-Feng Meng, Xin-Jiang Zhu, Guo Peng, Dong-Qiu Dai

Chun-Feng Meng, Xin-Jiang Zhu, Guo Peng, Dong-Qiu Dai, Department of Surgical Oncology, The First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China
Supported by National Natural Science Foundation of China, No. 30271477, No. 30572162
Correspondence to: Dr. Dong-Qiu Dai, Department of Surgical Oncology, The First Affiliated Hospital, China Medical University, No.155, Nanjing North Street, Heping District, Shenyang 110001, Liaoning Province, China. daidq63@163.com
Telephone: +86-24-83283560 Fax: +86-24-22703576
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Abstract

AIM: To identify the relationship between DNA hypermethylation and histone modification at a hypermethylated, silenced tumor suppressor gene promoter in human gastric cancer cell lines and to elucidate whether alteration of DNA methylation could affect histone modification.

METHODS: We used chromatin immunoprecipitation (ChIP) assay to assess the status of histone acetylation and methylation in promoter regions of the *p16* and *mutL homolog 1 (MLH1)* genes in 2 gastric cancer cell lines, SGC-7901 and MGC-803. We used methylation-specific PCR (MSP) to evaluate the effect of 5-Aza-2′-deoxycytidine (5-Aza-dC), trichostatin A (TSA) or their combination treatment on DNA methylation status. We used RT-PCR to determine whether alterations of histone modification status after 5-Aza-dC and TSA treatment are reflected in gene expression.

RESULTS: For the *p16* and *MLH1* genes in two cell lines, silenced loci associated with DNA hypermethylation were characterized by histone H3-K9 hypoacetylation and hypermethylation and histone H3-K4 hypomethylation. Treatment with TSA resulted in moderately increased histone H3-K9 acetylation at the silenced loci with no effect on histone H3-K9 methylation and minimal effects on gene expression. In contrast, treatment with 5-Aza-dC rapidly reduced histone H3-K9 methylation at the silenced loci and resulted in reactivation of the two genes. Combined treatment with 5-Aza-dC and TSA was synergistic in reactivating gene expression at the loci showing DNA hypermethylation. Similarly, histone H3-K4 methylation was not affected after TSA treatment, and increased moderately at the silenced loci after 5-Aza-dC treatment.

CONCLUSION: Hypermethylation of DNA in promoter CpG islands is related to transcriptional silencing of tumor suppressor genes. Histone H3-K9 methylation in different regions of the promoters studied correlates with DNA methylation status of each gene in gastric cancer cells. However, histone H3-K9 acetylation and H3-K4 methylation inversely correlate with DNA methylation status of each gene in gastric cancer cells. Alteration of DNA methylation affects histone modification.

Key words: Gastric cancer; DNA hypermethylation; Histone methylation; Histone acetylation; *p16*; *mutL homolog 1*; 5-Aza-2′-deoxycytidine; Trichostatin A

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INTRODUCTION

Multiple recent reviews have shown that virtually all human cancer types have epigenetic abnormalities that collaborate with genetic changes to drive cancer development and progression[1-7]. Hypermethylation of DNA in promoter CpG islands of tumor suppressor genes (TSGs) is known to inhibit transcriptional initiation and cause permanent silencing of the genes, which play a crucial role in carcinogenesis[1,2]. It was reported that hypermethylation of DNA in promoter CpG islands and diminished expression are present in a number of tumor-related genes in gastric cancer, which is one of the major current causes for cancer death in Asian countries[8]. For example, silencing of the cyclin-dependent kinase inhibitor *p16* gene induced by hypermethylation can lead to disruption of cell cycle regulation and provide a growth advantage to affected cells[9]. A mismatch repair

gene, *MLH1*, is often silenced with aberrant CpG island hypermethylation in gastric cancers[10,11]. Except for DNA methylation, recent studies have demonstrated the importance of histone modification as another epigenetic mechanism in the organization of chromosomal domains and gene regulation[12-18]. Acetylation of H3-K9 and methylation of H3-K4 are associated with open chromatin configurations such as that found at transcriptionally active promoters. In contrast, methylation of H3-K9 is a marker of condensed, inactive chromatin of the sort associated with the inactive X-chromosome and pericentromeric heterochromatin[16,17,19,20].

It has also been shown that histone modification is crucial to the process of DNA methylation in some organisms and abrogation of H3-K9 methylation in Neurospora results in loss of DNA methylation[21]. It was reported that histone H3-K9 methylation directly correlates with DNA methylation of some tumor suppressor genes, while histone H3-K9 acetylation and histone H3-K4 methylation inversely correlate with DNA methylation of some tumor suppressor genes[22]. These data suggest a functional linkage between DNA methylation and histone modifications in gene repression. To better understand the relationship between DNA methylation and histone modification in cancer-associated gene silencing, we performed ChIP assay to assess the methylation and acetylation of H3-K9 and the methylation of H3-K4 at the *p16* and *MLH1* genes in two gastric cancer cell lines. We also treated the gastric cancer cell lines with the DNA methylation inhibitor, 5-Aza-dC, and the histone deacetylase inhibitor, TSA, to elucidate whether alteration of DNA methylation affects histone modification.

MATERIALS AND METHODS

Cell lines and culture conditions

Two cell lines derived from human gastric cancer, SGC-7901 and MGC-803, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum(Gibco), penicillin (100 IU/mL) and streptomycin (100 μg/mL), and incubated in a humidified incubator containing 50 mL/L CO_2 at 37℃.

Treatment with 5-Aza-dC and TSA

TSA and 5-Aza-dC were purchased from Sigma. TSA was dissolved in absolute ethanol at a stock concentration of 3.3 mmol/L and stored at -80℃. 5-Aza-dC was dissolved in water at a stock concentration of 1 mmol/L and stored at -80℃. Cells were seeded at a low density in a 100 mm tissue culture dish and incubated for 24 h prior to treatment with 5-Aza-dC and TSA. 5-Aza-dC (5 μmol/L) was used for 72 h in the treatment. Culture medium containing 5-Aza-dC was exchanged every 24 h. TSA (300 nmol/L) was used for only 24 h in the treatment. 5-Aza-dC was used for 48 h followed by TSA for an additional 24 h in the combined treatment. Mock-treatment with an identical volume of absolute ethanol or water was used as a control.

Methylation-specific PCR

The genomic DNA was modified by bisulfite treatment, as described previously[23]. DNA was purified using a Wizard DNA clean-up system (Promega), precipitated with ethanol, and resuspended in 30 μL of Tris-EDTA buffer. Two microliters of the aliquot was used as a template. The primers used for MSP and additional PCR conditions are described elsewhere[22]. PCR products were separated by electrophoresis on 2% agarose gels and quantitated with the FluorChem 2.0 system. The experiment was repeated three times.

RT-PCR analysis of p16 and MLH1 expression

Total cellular RNA was extracted from each of the two cell lines with TriZOL (Invitrogen) according to the manufacturer's protocol. RNA was resuspended in nuclease-free water and quantitated with a spectrophotometer. Reverse transcription (RT) reactions were done on 2 μg of total RNA following the manufacturer's protocol (Promega). cDNA was amplified by PCR using primers as described previously. Reaction conditions for each PCR are described elsewhere[24]. PCR products were resolved on 2% agarose gels and quantitated using the Fluor Chem 2.0 system. The level was determined by quantifying the intensities of the PCR product versus *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). The experiment was repeated three times.

Chromatin immunoprecipitation assay

ChIP assays were performed as described previously with some modifications[24]. Briefly, proteins were cross-linked to DNA by adding formaldehyde directly into the culture medium to a final concentration of 4 g/L for 20 min at 37℃. After washing, the cell pellets were resuspended in 500 μL lysis buffer and sonicated thirty-five times, 2 s each. The average fragment size after sonication was 500 bp. The lysate (500 μL) was then divided into three fractions. The first and second fractions (200 μL each) were diluted in 1800 μL of lysis buffer, and the third fraction (100 μL) was used as an input control. The first lysate was incubated overnight at 4℃ with 5 μL anti-Lys-9 acetylated histone H3 antibody, 5 μL anti-Lys-9 dimethylated histone H3 antibody, or 5 μL anti-Lys-4 dimethylated histone H3 antibody (all antibodies from Upstate Biotechnology) overnight at 4℃. The second lysate was incubated with Tris-EDTA buffer (5 μL) as a negative control. Immune complexes were collected with 20 μL protein A-sepharose beads for 1 h at 4℃ with agitation. The cross-links were reversed by heating the sample at 65℃ for 5 h. After elution, the samples were digested with proteinase K. DNA was recovered by phenol extraction, precipitated with ethanol, and resuspended in Tris-EDTA buffer.

PCR analysis of immunoprecipitated DNA

Amplification was carried out with 2 μL of an immunoprecipitated DNA, a control without antibody or a 1:10 dilution of input DNA that was not immunoprecipitated. The primers used for ChIP and PCR conditions are described elsewhere[22]. We selected *P16-3, P16-6, MLH1-2* and *MLH1-3*. PCR products were electrophoresed on 2% agarose gels and quantitated with the FluorChem 2.0 system. The level of histone acetylation and methylation in each immunoprecipitation was measured by quantifying the intensities of the PCR product in

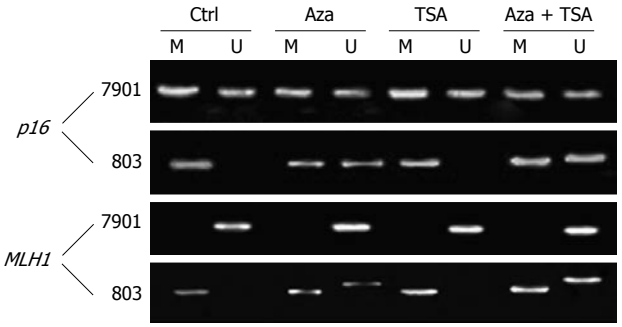

Figure 1 MSP analysis for promoter regions of gastric cancer cells after treatment with 5-Aza-dC, TSA or their combination. M: Methylated alleles; U: Unmethylated alleles; Ctrl: No treatment.

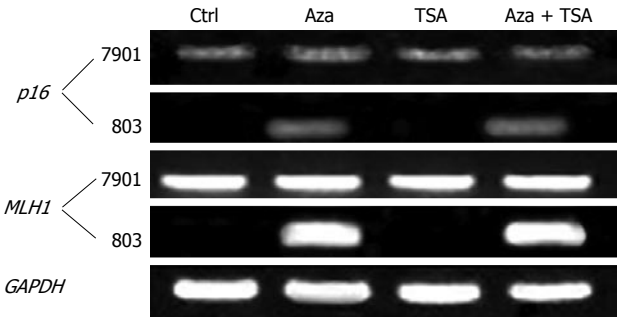

Figure 2 Expression and reactivation of *p16* and *MLH1* in gastric cancer cells after treatment with 5-Aza-dC, TSA or their combination. Ctrl: No treatment.

immunoprecipitated DNA versus input DNA diluted at 1:10 (total chromatin). The experiment was repeated three times.

RESULTS

MSP analysis for each promoter region after treatment with 5-Aza-dC, TSA or their combination

The two cell lines showed a characteristic DNA methylation status in each promoter region. As shown in Figure 1, *p16* was hypermethylated (both alleles methylated) in MGC-803 and partially methylated (only one allele methylated) in SGC-7901. *MLH1* was hypermethylated in MGC-803 but not methylated in SGC-7901.

5-Aza-dC and combined 5-Aza-dC and TSA resulted in demethylation of *p16* and *MLH1* in MGC-803, in which the silenced gene was associated with DNA hypermethylation. In contrast, TSA alone did not affect the DNA methylation status of *p16* and *MLH1*.

RT-PCR analysis for expression and reactivation of p16 and MLH1 after treatment with 5-Aza-dC, TSA or their combination

As shown in Figure 2, *p16* was expressed in SGC-7901 and minimally affected by TSA. *p16* was silenced in MGC-803 and TSA was not able to activate gene expression. In contrast, 5-Aza-dC alone reactivated expression of the *p16* in MGC-803. Similar results were obtained in *MLH1*, which was expressed in SGC-7901 but silenced in MGC-803. TSA had no effect on gene expression, while 5-Aza-dC reactivated the silenced gene. The combined

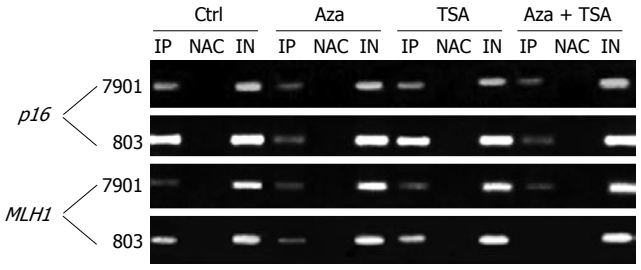

Figure 3 Status of histone H3-K9 methylation across TSGs and change in gastric cancer cells after treatment with 5-Aza-dC, TSA or their combination. Ctrl: No treatment; IP: Immunoprecipitated DNA; NAC: No-antibody control; IN: Input DNA from whole-cell lysate.

treatment with 5-Aza-dC and TSA increased gene expression.

ChIP assay for histone H3-K9 methylation across the promoter of TSG and change after treatment with 5-Aza-dC, TSA or their combination

The results of ChIP studies were almost identical in different regions of each promoter, and the values for each gene were averaged to present the data. In the promoter region of the *p16* gene, H3-K9 methylation was higher for MGC-803 than for SGC-7901. Similar results were seen at *MLH1*. SGC-7901 having no promoter DNA methylation at this locus, showed a low degree of H3-K9 methylation. MGC-803 had a higher degree of H3-K9 methylation across the promoter (Figures 3 and 4).

TSA alone had no effect on H3-K9 methylation, irrespective of DNA methylation status. In contrast, 5-Aza-dC had effects on H3-K9 methylation at the silenced loci, reducing histone H3-K9 methylation in the promoter showing partial methylation or hypermethylation (the promoter region of *p16* in both cell lines and the promoter region of *MLH1* in MGC-803). The combination of 5-Aza-dC and TSA had similar effects on histone H3-K9 methylation.

ChIP assay for histone H3-K9 acetylation across the promoter of TSG and change after treatment with 5-Aza-dC, TSA or their combination

The promoter region of the *p16* gene showed a higher degree of H3-K9 acetylation in SGC-7901 (partially methylated) than in MGC-803 (hypermethylated). Similar results were seen in the *MLH1* gene showing a low degree of H3-K9 acetylation in all parts of the promoter region in MGC-803. In contrast, a higher degree of H3-K9 acetylation was detected in SGC-7901 (both alleles non-methylated) at all *MLH1* regions studied (Figures 4 and 5A).

For the *p16* gene, treatment with TSA alone had no effect on H3-K9 acetylation in the SGC-7901 (partial DNA methylation) but slightly increased H3-K9 acetylation in the silenced MGC-803. Identical results were seen in *MLH1*. 5-Aza-dC increased H3-K9 acetylation at the loci with DNA hypermethylation (*p16* and *MLH1* in MGC-803) but had no effect on the loci with partial or no DNA methylation (*p16* and *MLH1* in BGC-7901). However, the combination of 5-Aza-dC and TSA increased H3-K9

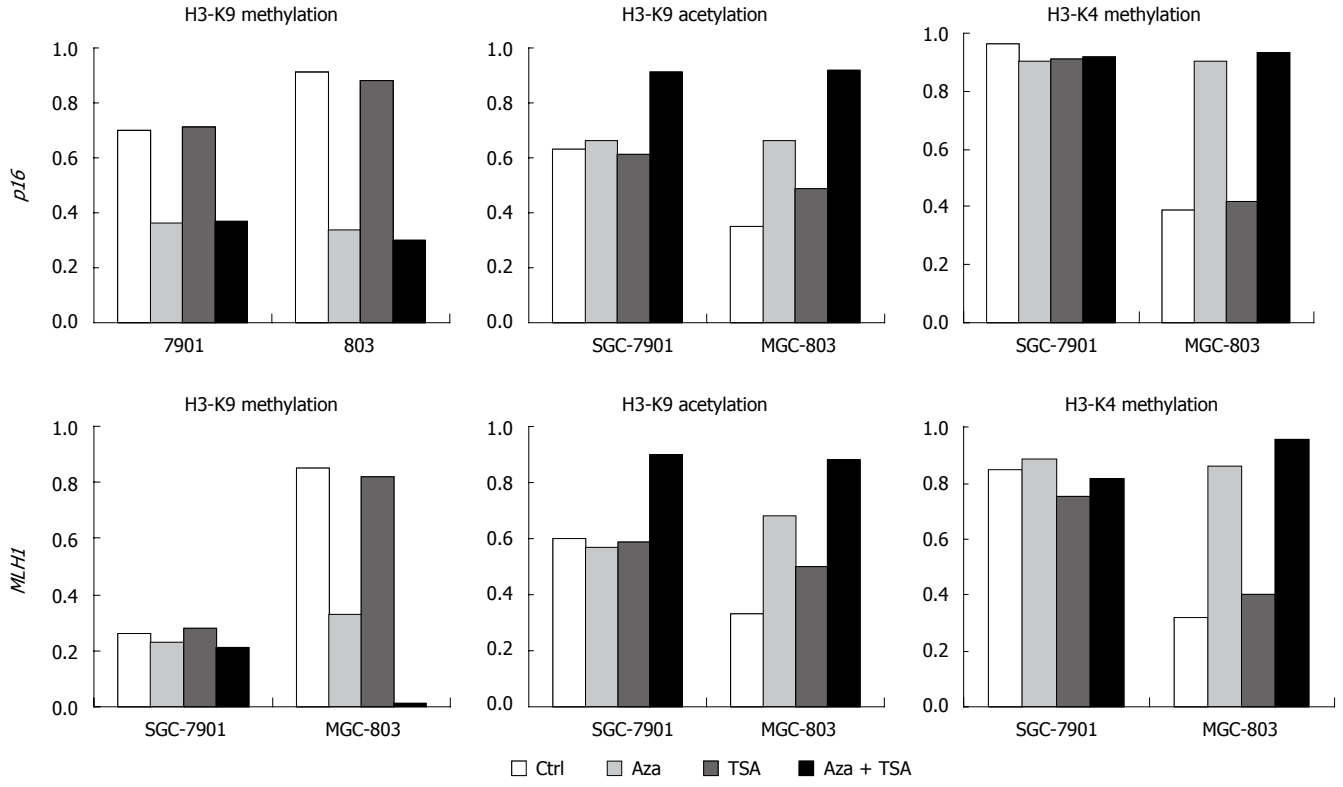

Figure 4 Summary of quantitative analysis for ChIP assays. Ratios of precipitated DNA over input DNA were used to calculate the relatively precipitated fold enrichment on the y axis.

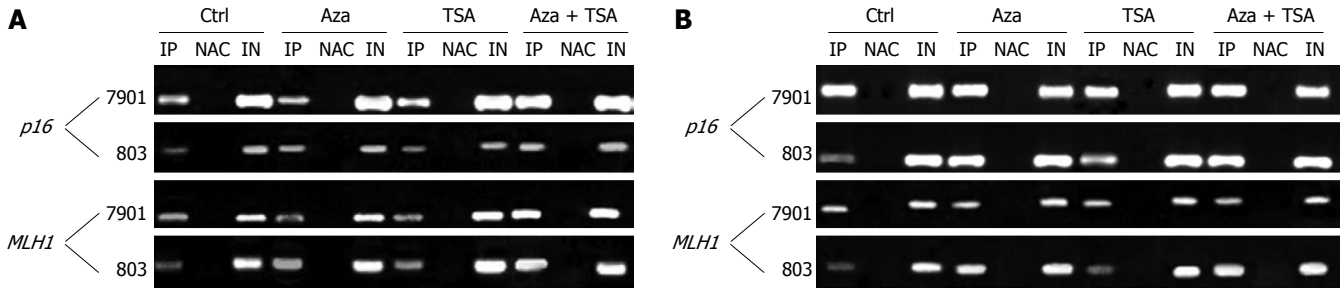

Figure 5 Status of histone H3-K9 acetylation (**A**) and histone H3-K4 methylation (**B**) across TSGs and change in gastric cancer cells after treatment with 5-Aza-dC, TSA or their combination. Ctrl: No treatment; IP: Immunoprecipitated DNA; NAC: No-antibody control; IN: Input DNA from whole-cell lysate.

acetylation effectively at all loci irrespective of DNA methylation status. The results of ChIP studies were almost identical in different regions of each promoter.

ChIP assay for histone H3-K4 methylation across the promoter of TSG and change after treatment with 5-Aza-dC, TSA or their combination

For the *p16* and *MLH1* genes, H3-K4 methylation was higher in SGC-7901 than in MGC-803. TSA did not affect H3-K4 methylation. However, 5-Aza-dC, or the combination of 5-Aza-dC and TSA, increased H3-K4 methylation at all silenced loci in MGC-803. Little change in H3-K4 methylation was observed in SGC-7901, where only partial or no methylation was observed (Figures 4 and 5B).

DISCUSSION

Silencing of tumor suppressor genes is related to epigenetic regulation of both DNA methylation and histone modification[25]. In the present study, hypermethylation in promoter CpG islands was significantly associated with *p16* and *MLH1* silencing. Furthermore, aberrantly silenced and DNA hypermethylated genes in gastric cancer cells were characterized by histone H3-K9 hypermethylation, H3-K4 hypomethylation and H3-K9 hypoacetylation.

DNA methylation and histone modification may act synergistically or antagonistically on gene expression[26,27]. We carried out ChIP assays to explore the relationship between DNA methylation and histone modifications. ChIP is a powerful technique to test for the presence of certain DNA-binding proteins that might modulate chromatin structure and/or transcriptional characteristics of the specific region of DNA with which they are associated. We made use of polyclonal antibodies generated against methylated and acetylated histone H3, all of which are proteins linked to chromatin modification

and regulation of transcription. In colorectal cancer, histone H3-K9 methylation directly correlates and histone H3-K4 methylation inversely correlates with DNA methylation of *p16, MLH1* and the O6-methylguanine-DNA methyltransferase gene, *MGMT*[22]. We demonstrated that histone H3-K9 methylation correlated, and histone H3-K9 acetylation and H3-K4 methylation inversely correlated very well with DNA methylation of *p16, MLH1* in SGC-7901 and MGC-803.

To further explore the relationship between DNA methylation and histone modification, we treated cancer cells with 5-Aza-dC and TSA. 5-Aza-dC, a DNA methyltransferase inhibitor, is sufficient to cause demethylation of the promoter region and reactivate expression of the hypermethylated, silenced gene[28,29]. TSA, a specific histone deacetylase (HDAC) inhibitor, has been permitted evaluation of the role of HDAC in silencing a variety of methylated genes[14]. It was reported that re-expression of DNA hypermethylated and silenced cancer genes can be induced through 5-Aza-dC-induced DNA demethylation, demethylated genes and the active marks, acetylated H3-K9 and methylated H3-K4 can be detected in HCT116 and DKO colon cancer[29-34]. However, one silencing mark, dimethylated H3-K9, is strikingly decreased[34]. In our study, when the CpG islands were hypermethylated, TSA increased histone acetylation, but had almost no effect on gene expression. In contrast, 5-Aza-dC reactivated expression of hypermethylation-induced silenced genes. Our findings on histone acetylation are consistent with previous reports linking the effect of DNA methylation and histone deacetylation on transcriptional silencing, demonstrating that DNA methylation is dominant over histone deacetylation in maintaining a silent state at hypermethylated promoters[22]. Furthermore, TSA and 5-Aza-dC play a different role in histone methylation. In the present study, 5-Aza-dC, but not TSA, could reactivate expression of the silenced genes and completely reverse key histone methylations surrounding the gene promoter, indicating that reactivation of silenced genes correlates much better with decreased histone H3-K9 methylation and increased H3-K4 methylation than with increased H3-K9 acetylation. We speculate that histone methylation plays a critical role in the maintenance of promoter DNA methylation-associated gene silencing in gastric cancer.

After 5-Aza-dC treatment, we observed a complete reversal of histone modification at the *p16* and *MLH1* promoter in MGC-803 cells. Acetylated H3-K9 and methyl-H3-K4 levels were increased, whereas methyl-H3-K9 levels decreased, suggesting that DNA hypermethylation may be essential for maintaining histone modification at gene promoters silenced due to aberrant DNA hypermethylation. DNA methylation plays a direct role in both genes silencing and maintaining a repressive histone modification at a hypermethylated gene promoter in cancer. Data show that DNMT1 interacts with HDAC activity in complexes bound to DNA, suggesting that it can recruit histone modifiers to DNA[33-35]. It was reported that DNA modification itself, or components of the DNA methylating machinery such as DNMTs or methyl-CpG binding proteins, can directly interact with histone methyltransferases or proteins in regions containing DNA methylation and allow them to set up an alterative histone modification[2], showing that histone methylation depends on DNA methylation.

COMMENTS

Background

Gastric cancer is a malignant tumor threatening human health worldwide. It has been shown that epigenetic mechanism plays an important role in the occurrence and development of gastric cancer. However, the role of histone modification and the relationship between DNA hypermethylation and histone modification at a hypermethylated, silenced tumor suppressor gene promoter in human gastric cancer remain unclear.

Research frontiers

Histone modification plays a prominent role in the epigenetic regulation of gene transcription. There is evidence that dysregulation of epigenetic process causes transcriptional repression of a subset of genes, contributing to the pathogenesis of many cancers. The relationship between aberrant epigenetic changes and tumorigenesis is still to be identified.

Innovations and breakthroughs

In some cancers, histone H3-K9 methylation directly correlates and histone H3-K4 methylation inversely correlates with DNA methylation of some TSGs. Our findings may reflect the mechanism underlying inactivation of tumor suppressor genes by DNA hypermethylation and histone modification during gastric tumorigenesis. DNA methylation may affect histone modification in human gastric cancer.

Applications

Reactivation of the *p16* and *MLH1* gene by DNA methyltransferase inhibitor alone or in combination with histone deacetylase inhibitor suggests that the two agents can be used in treatment of gastric cancer.

Terminology

Histone is the major component of chromatin functioning as a DNA packaging unit and as a transcriptional regulator. The amino-terminal tails of histones protrude from the nucleosome and are subjected to chemical modifications including phosphorylation, acetylation and methylation. These modifications of histones affect the access of regulatory factors and complexes to chromatin and influence gene expression. These different combinations of histone modifications at different residues may act synergistically or antagonistically on gene expression.

Peer review

This is an important and interesting study, and the manuscript is well-written. The major finding in this paper is that treatment with 5-Aza-dC, a DNA methylation inhibitor and TSA, a histone deacetylase inhibitor, affects the expression of tumor suppressor genes, including *p16* and *MLH1*, by reversing the hypermethylation and inhibiting histone deacetylation in human gastric cancer cells.

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BASIC RESEARCH

Protective effects of erythropoietin against acute lung injury in a rat model of acute necrotizing pancreatitis

Oge Tascilar, Güldeniz Karadeniz Cakmak, Ishak Ozel Tekin, Ali Ugur Emre, Bulent Hamdi Ucan, Burak Bahadir, Serefden Acikgoz, Oktay Irkorucu, Kemal Karakaya, Hakan Balbaloglu, Gürkan Kertis, Handan Ankarali, Mustafa Comert

Oge Tascilar, Güldeniz Karadeniz Cakmak, Ali Ugur Emre, Bulent Hamdi Ucan, Oktay Irkorucu, Kemal Karakaya, Hakan Balbaloglu, Mustafa Comert, Department of Surgery, Zonguldak Karaelmas University, School of Medicine, Kozlu-Zonguldak 67600, Turkey
Ishak Ozel Tekin, Department of Immunology, Zonguldak Karaelmas University, School of Medicine, Kozlu-Zonguldak 67600, Turkey
Burak Bahadir, Gürkan Kertis, Department of Pathology, Zonguldak Karaelmas University, The School of Medicine, Kozlu-Zonguldak 67600, Turkey
Serefden Acikgoz, Department of Biochemistry, Zonguldak Karaelmas University, School of Medicine, Kozlu-Zonguldak 67600, Turkey
Handan Ankarali, Department of Biostatistics, Zonguldak Karaelmas University, School of Medicine, Kozlu-Zonguldak 67600, Turkey
Correspondence to: Dr. Güldeniz Karadeniz Cakmak, Zonguldak Karaelmas Universitesi, Arastirma ve Uygulama Hastanesi Bashekimligi, Kozlu-Zonguldak 67600, Turkey. gkkaradeniz@yahoo.com
Telephone: +90-372-2610169 Fax: +90-372-2610155
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Abstract

AIM: To investigate the effect of exogenous erythropoietin (EPO) administration on acute lung injury (ALI) in an experimental model of sodium taurodeoxycholate-induced acute necrotizing pancreatitis (ANP).

METHODS: Forty-seven male Wistar albino rats were randomly divided into 7 groups: sham group (n = 5), 3 ANP groups (n = 7 each) and 3 EPO groups (n = 7 each). ANP was induced by retrograde infusion of 5% sodium taurodeoxycholate into the common bile duct. Rats in EPO groups received 1000 U/kg intramuscular EPO immediately after induction of ANP. Rats in ANP groups were given 1 mL normal saline instead. All animals were sacrificed at postoperative 24 h, 48 h and 72 h. Serum amilase, IL-2, IL-6 and lung tissue malondialdehyde (MDA) were measured. Pleural effusion volume and lung/body weight (LW/BW) ratios were calculated. Tissue levels of TNF-α, IL-2 and IL-6 were screened immunohistochemically. Additionally, ox-LDL accumulation was assessed with immune-fluorescent staining. Histopathological alterations in the lungs were also scored.

RESULTS: The mean pleural effusion volume, calculated LW/BW ratio, serum IL-6 and lung tissue MDA levels were significantly lower in EPO groups than in ANP groups. No statistically significant difference was observed in either serum or tissue values of IL-2 among the groups. The level of tumor necrosis factor-α (TNF-α) and IL-6 and accumulation of ox-LDL were evident in the lung tissues of ANP groups when compared to EPO groups, particularly at 72 h. Histopathological evaluation confirmed the improvement in lung injury parameters after exogenous EPO administration, particularly at 48 h and 72 h.

CONCLUSION: EPO administration leads to a significant decrease in ALI parameters by inhibiting polymorphonuclear leukocyte (PMNL) accumulation, decreasing the levels of proinflammatory cytokines in circulation, preserving microvascular endothelial cell integrity and reducing oxidative stress-associated lipid peroxidation and therefore, can be regarded as a cytoprotective agent in ANP-induced ALI.

Key words: Erythropoietin; Acute pancreatitis; Acute lung injury; Acute respiratory distress syndrome; Cytokine

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INTRODUCTION

Acute pancreatitis (AP) is a life-threatening necro-inflammatory disease with significant morbidity and mortality rates, especially when complicated by systemic inflammatory response syndrome (SIRS) and multiple organ failure (MODS)[1,2]. Death occurs in 60% of the patients within the first 6 d of disease onset and pulmonary complications including acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)

account for a significant number of these deaths[3]. The exact mechanisms by which diverse etiological factors induce an attack are indefinite, but once the disease process is initiated, common inflammatory and repair pathways are invoked. Within the first few days following the onset of AP, lung injury occurs as a consequence of AP, whereas sepsis is a dominant cause for lung injury and mortality in the later phase of the disease process[4]. Despite improved understanding of the pathogenesis of ARDS, pharmacological modalities are ineffective in decreasing its mortality. None of the randomized clinical trials using novel therapeutic agents has demonstrated an improvement in patient outcome. Consequently, effective therapeutic interventions are thus called for.

Erythropoietin (EPO), a 30.4-kDa glycoprotein and a member of the type I cytokine superfamily, was first introduced as a hormone that regulates erythroid progenitors within the bone marrow to mature into erythrocytes, through binding to its specific cell surface receptors[5]. Hence, EPO is approved for the treatment of anemia as a consequence of a variety of disorders. In the current era, the premise that EPO is essential only for erythropoiesis has been changed according to the researches demonstrating the existence of EPO and its receptor in other organs and tissues outside of liver and kidney, such as brain, heart, pancreas, as well as vascular, gastrointestinal and reproductive systems[6,7]. Beyond its hematopoietic properties, EPO modulates a broad array of vital cellular processes including progenitor stem cell development, cellular integrity, and angiogenesis[8,9]. Additionally, in various tissues, EPO inhibits the apoptotic mechanisms of injury, including preservation of cellular membrane asymmetry to prevent inflammation[10-12]. Experimental evidence supports a vigorous cytoprotective effect and EPO is now considered to have applicability in a variety of disorders, such as cerebral ischemia, myocardial infarction, and chronic congestive heart failure[12-15]. Wu *et al*[16] demonstrated that pretreatment with EPO appears to attenuate ischemia-reperfusion-induced lung injury. This function is partly related with the ability of EPO to inhibit the accumulation of polymorphonuclear leukocytes (PMNL) in lung tissue and decrease the systematic expression of tumor necrosis factor-α (TNF-α). In addition to these studies, it has been reported that EPO can attenuate different kinds of lung injuries, showing that rats exposed to hyperoxia exhibit well-maintained alveolar structure and enhanced vascularity when treated with EPO[17]. Importantly, EPO can protect the ultrastructure of tracheobronchial epithelia and pulmonary type Ⅱ epithelia of rats during traumatic brain injury[18,19].

AP associated lung injury is a multifactorial phenomenon with various phases. In the light of the above-mentioned findings, the present study was to evaluate the hypothesis that EPO administration offers pulmonary protective effect against pancreatitis induced lung injury in rats.

MATERIALS AND METHODS

Animals

Forty-seven male Wistar albino rats weighing 250-300 g were housed under constant temperature (22℃) and humidity in a 12-h dark/light cycle.

Experimental design

The experiments were conducted following the Ethic Committee Faculty of Medicine, University of Zonguldak Karaelmas guiding principles for the care and use of laboratory animals. The animals were randomized into seven experimental groups as follows: sham group in which rats received sham operation (n = 5), 3 ANP groups in which acute necrotizing pancreatitis (ANP) was induced by retrograde infusion of sodium taurodeoxycholate and 1 mL normal saline (0.9% NaCl) was given intramuscularly immediately after induction of AP (n = 7 each), 3 EPO groups in which AP was induced by the same way and 1000 U/kg EPO (Eprex, Epoetin alfa, Janssen-Cilag AG, Sweden) was injected intramuscularly immediately after induction of AP. All animals in the ANP and EPO groups were sacrificed at postoperative 24 h, 48 h and 72 h, respectively. Histopathological, biochemical and immunohistochemical evaluations were performed.

Induction of acute pancreatitis

Anesthesia was induce by injecting ketamine HCL at 100 mg/kg im and laparotomy was performed under strict sterile conditions. An upper midline abdominal incision was made to identify the common pancreaticobiliary duct. The duodenal wall was punctured at its antimesenteric aspect with a 24-gauge Ⅳ catheter (Novacath, Medipro A.Ş., Istanbul, Turkey). The catheter was advanced 5 mm into the common duct through the papilla of Vater. ANP was induced by retrograde infusion of 0.2 mL 5% sodium taurodeoxycholate (Sigma, St.Louis, MO, USA) over 3 min using an infusion pump as previously described[20] and the pacreaticobiliary duct was clamped near the liver hilum throughout the inraductal infusion in all groups, except for sham group. Animals in sham group were subjected to anesthesia, laparotomy and duodenal manipulation, but not to biliopancreatic duct cannulation. The midline incision was closed in two layers with 4/0 silk suture (Ethicon, Edinburg, UK). Rats were allowed to recover from anesthetic and returned to their cages with free access to water and food after surgery.

Sampling procedures

All the rats were sacrificed by aortic puncture method. The abdominal and thoracic cavities were entered to obtain blood and lung samples. Blood samples were centrifuged at 1800 × g for 15 min at 4℃ to obtain plasma and stored at -80℃ for biochemical analysis. Then, the rats were killed with the lung removed immediately. Random cross-sections of the lung tissue were fixed in 10% neutral phosphate-buffered formalin and embedded in paraffin wax for histopathological examination. Samples of lung tissue were weighed and stored at -85℃ for subsequent biochemical and immunohistochemical measurements.

Assessment of pulmonary effusion

The thorax was opened to collect pleural effusion (PE) by suction which was measured volumetrically. Care was also taken to eliminate blood contamination with PE. The

lungs were then removed and all surrounding tissues were dissected and weighed with an analytical balance. The volume of PE (mL) and the lung weight/body weight (LW/BW) ratios were calculated and considered as an index of pulmonary edema.

Biochemical analysis

Serum amylase, IL-2 and IL-6 assay: Serum amylase levels were measured by a Beckman Coulter LX-20® system analyzer (Fullerton, CA, USA) using Beckman kits (Fullerton, CA, USA), following the manufacturer's instructions. IL-2 and IL-6 levels in the serum were measured with commercially available kits (Biosource International, Commercial ELISA Kit, California, USA).

Lung tissue malondialdehyde (MDA) assay: MDA levels in the lung tissue were measured in tissue homogenate. In brief, tissue was homogenized with cold 1.15% KCl to make a 10% homogenates, and 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 with NaOH and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid were added to 0.2 mL of 10% tissue homogenates. The mixture was made up to 4.0 mL with distilled water and heated in an oil bath at 95℃ for 60 min. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, v/v) were added and the solution was shaken vigorously. After centrifugation at 4000 r/min for 10 min, the organic layer was taken with its absorbance measured at 532 nm on a Shimadzu UV 1601 spectrophotometer. As a standard, 1.1.3.3 tetraetoxypropane was used. MDA concentration per gram tissue was calculated (nmol/gr tissue).

Immunohistochemical method for screening IL-2, IL-6 and TNF-α in the lung tissue: Cryostat sections of lung tissue (7 μm) were fixed with absolute ethanol and stained with avidin biotin complex based immunohistochemical method. Immunohistochemistry was performed to observe peroxidase diaminobenzidine reaction. Cytokine staining was performed with biotinylated mouse anti-rat IL-2, IL-6, TNF-α antibodies (Biosource International, California, USA). Streptavidin-peroxidase (HRP) and diaminobenzidine (DAB) were purchased from DAKOCytomation (Denmark). Ethanol-fixed tissue sections were treated with biotinylated mouse anti-rat IL-2, IL-6 and TNF-α for 30 min, washed three times with PBS, incubated for an additional 30 min with streptavidin-HRP and washed three times with PBS. The sections were then treated with 0.03% 3, 3-diamino benzidine tetrahydrochloride plus 0.01% hydrogen peroxide in 50 mmol/L Tris-HCl buffer (pH 7.4) for 10 min. All incubations were performed at room temperature. The sections were examined under a light microscope by an independent observer, blind to the study.

Immune-fluorescent staining method for screening ox-LDL in the pancreas and lung tissues: Rat lung and pancreas were obtained and stored at -85℃. Slides were prepared from the 7 μm-thick frozen lung biopsy sections. Slides were further divided into two pieces: one for the test and the other for the negative control. Thirty μL human polyclonal anti-ox-LDL IgG solution as primary antibody was added only on the test slides and the control slides were manipulated with phosphate- buffered solution (PBS) as the same amount of primary antibody. After a 30-min incubation in a humid chamber at room temperature, both the control and test slides were washed with phosphate-buffered saline and 30 μL fluorescent isothiocyanate (FITC)-labeled anti human IgG was administered as a conjugate substance. The slides were incubated for a further 30 min at room temperature and washed with the standard PBS solution. After drying, the slides were covered with a mounting medium and examined under a fluorescent microscope (LEICA DMRX, Germany).

Histopathologic analysis

The lung tissue samples were fixed in 10% formalin immediately after removal, embedded in paraffin, sectioned at 5 μm intervals, stained with hematoxylin and eosin, and examined under a light microscope. Histopathological evaluation and scoring of the parameters were performed by a single pathologist unaware of the treatment groups. Morphometric analysis of histological sections was accomplished with the point counting technique. For this purpose, we used an optical microscope provided with an integrating eyepiece containing 100 points and 50 lines. The following parameters were evaluated as previously described[21,22].

Alveolar distension and collapse index: At a magnification of × 100, we analyzed 10 randomly selected fields of the proximal and 10 fields of the distal sections. We designated grades 0, 1, 2, and 3 to microscopic fields respectively as 0%, 25%, 50%, and over 50% of the area with either alveolar distension or alveolar collapse.

Alveolar edema index: At a magnification of × 400, we analyzed 10 randomly selected fields of the proximal and 10 fields of the distal sections. The relationship between the number of points of the eyepiece falling on alveolar edema and the number of points falling on the whole alveolar lumen was determined.

Alveolar cellularity index: We analyzed 10 microscopic fields from each lung slide at a magnification of × 1000. The alveolar cellularity index was obtained by the relationship between the lines of the integrating eyepiece crossing a nucleus and the lines crossing alveolar septa.

Polymorphonuclear cell (PMNL) index: We analyzed 10 microscopic fields from each lung slide at a magnification of × 1000. PMNL index was obtained by the relationship between the lines of the integrating eyepiece crossing a nucleus and the lines crossing alveolar septa.

Statistical analysis

Statistical analysis was performed using SPSS version 11.5 for Windows XP. The results were expressed as mean + standard deviation (SD). The differences in serum amylase, IL-2 and IL-6 were assessed by Welch test and *post hoc* Games-Howell test or one way ANOVA and *post hoc* Tukey

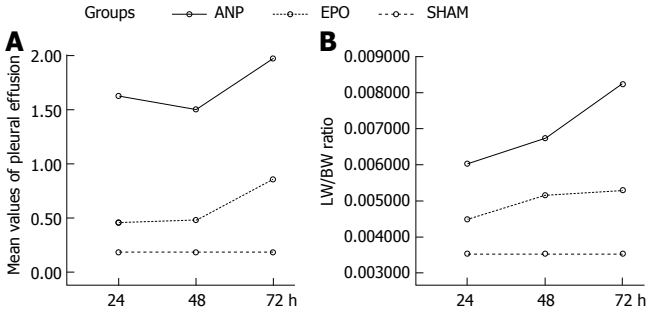

Figure 1 Values of pleural effusion volumes (**A**) and calculated LW/BW ratios (**B**) (mean ± SD).

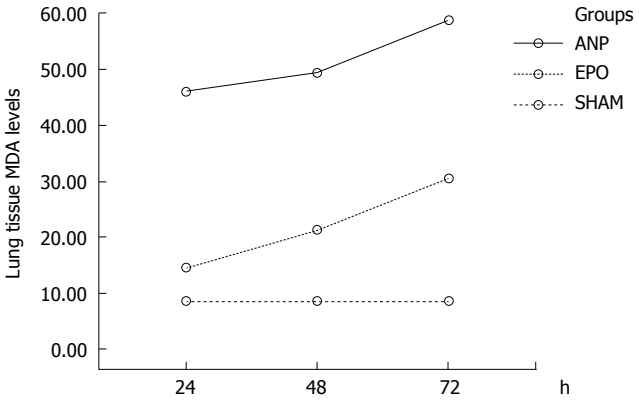

Figure 2 Values of MDA in lung tissue (mean ± SD).

HSD test where appropriate. The differences betweeen groups (ANP, EPO, sham), time course (three different hours) and its interaction in terms of tissue MDA levels, pulmonary effusion volume, calculated LW/BW ratio, and mean histopathological scores, were analyzed by factorial analysis of variance with a single control. $P < 0.05$ was considered statistically significant.

RESULTS

Pleural effusion and LW/BW ratio

The mean pleural effusion volume (mL) and the calculated LW/BW ratio were significantly increased in ANP groups when compared to EPO groups ($P < 0.0001$). The mean ± SD volume of pleural effusion measured was 1.62 ± 1.08 mL, 1.5 ± 0.33 mL and 1.97 ± 0.39 mL in 3 ANP groups and 0.45 ± 0.37 mL, 0.48 ± 0.38 mL and 0.85 ± 0.13 mL in 3 EPO groups, respectively. No statistically significant difference was detected between sham and EPO groups (0.18 ± 0.08 mL *vs* 0.45 ± 0.37 mL, 0.48 ± 0.38 mL and 0.85 ± 0.13 mL, $P > 0.05$ for each). The volume of pleural effusion was statistically significant higher in ANP groups than in sham group (0.18 ± 0.08 mL *vs* 1.62 ± 1.08 mL, 1.5 ± 0.33 mL and 1.97 ± 0.39 mL, $P < 0.001$ for each).The time course of pleural effusion volume in 3 ANP groups is shown in Figure 1A. In terms of LW/BW ratio, a statistically significant difference was seen from 24 h to 72 both in 3 ANP groups (0.006 ± 0.0022 *vs* 0.008 ± 0.0019, $P < 0.05$) and in 3 EPO groups (0.004 ± 0.0008 *vs* 0.005 ± 0.0011, $P < 0.05$) and the mean calculated ratio was higher at 72 h for each. In comparison to sham group, no statistically significant difference was found in EPO groups (0.003 ± 0.0004 *vs* 0.004 ± 0.0008, 0.005 ± 0.0009 and 0.005 ± 0.0011, $P > 0.05$), where as ANP resulted in a significant increase in calculated LW/BW ratio at 24 h, 48 h, and 72 h (0.003 ± 0.0004 *vs* 0.006 ± 0.0022, 0.007 ± 0.0016, and 0.008 ± 0.0019, $P < 0.05$ for each) (Figure 1B). The pleural effusion values and LW/BW ratio are listed in Table 1.

Biochemical analysis

Serum amylase, IL-2 and IL-6 assay: A statistically significant increase was detected in the mean ± SD serum levels of amylase in 3 ANP groups when compared to sham group (534 ± 124 u/L *vs* 3502 ± 1830 u/L, 3759 ± 1505 u/L and 5056 ± 1872 u/L, $P < 0.05$ for each). The mean ± SD value of serum amylase was 3502 ± 1830 u/L, 3759 ± 1505 u/L and 5056 ±1872 u/L in 3 ANP groups and 1523 ± 514 u/L, 2317 ± 311 u/L and 735 ± 454 u/L in 3 EPO groups, respectively. On the other hand, no statistically significant difference was found between the ANP and EPO groups with respect to the time intervals ($P > 0.05$). The serum levels of IL-6 were significantly lower in 3 EPO groups than in 3 ANP groups ($P = 0.001$ for each). The IL-6 value (mean ± SD) was 24.4 ± 3.26 pg/mL, 27.7 ± 3.74 pg/mL and 33.2 ± 2.1 pg/mL respectively in 3 ANP groups, and 12.2 ± 2.15 pg/mL, 12.8 ± 1.89 pg/mL and 13.8 ± 3.24 pg/mL respectively in 3 EPO groups. We did not observe any statistically significant difference in IL-2 values among these groups (7.4 ± 2.88 pg/mL *vs* 7.7 ± 3.17 pg/mL, 9.3 ± 2.74 pg/mL, 7.2 ± 3.1 pg/mL, 6.9 ± 2.04 pg/mL, 7.7 ± 2.93 g/mL and 10.6 ± 3.9 pg/mL, $P > 0.005$ for each). The mean ± SD values of serum amylase, IL-2, IL-6 and tissue MDA are listed in Table 2.

Lung tissue MDA assay: Pulmonary injury in ANP groups was characterized by an increase in lung tissue MDA levels, an indicator of lipid peroxidation. The lung tissue MDA levels were significantly reduced in EPO groups at 24 h, 48 h, and 72 h, when compared to ANP groups ($P < 0.0001$). The MDA value (mean ± SD) was 45.9 ± 6.8 nmol/gr tissue, 49.3 ± 9.5 nmol/gr tissue, and 58.8 ± 9 nmol/gr tissue respectively in 3 ANP groups and 14.7 ± 2.1 nmol/gr tissue, 21.2 ± 2.7 nmol/gr tissue, and 30.4 ± 2.1 nmol/gr tissue respectively in 3 EPO groups. A statistically significant increase in MDA values was noted at 24 h-72 h (45.9 ± 6.8 nmol/gr tissue *vs* 58.8 ± 9 nmol/gr tissue, and 14.7 ± 2.1 nmol/gr tissue *vs* 30.4 ± 2.1 nmol/gr tissue, $P < 0.0001$) and 48 h to 72 h (49.3 ± 9.5 nmol/gr tissue *vs* 58.8 ± 9 nmol/gr tissue and 21.2 ± 2.7 nmol/gr tissue *vs* 30.4 ± 2.1 nmol/gr tissue, $P = 0.001$) in either ANP or EPO groups. The mean MDA value was higher at 72 h. In comparison with sham group, the MDA levels were significantly higher in all the other groups (8.5 ± 3.1 nmol/gr tissue *vs* 45.9 ± 6.8 nmol/gr tissue, 49.3 ± 9.5 nmol/gr tissue, 58.8 ± 9 nmol/gr tissue, 21.2 ± 2.7 nmol/gr tissue, and 30.4 ± 2.1 nmol/gr tissue, $P < 0.001$ for each) except for EPO groups at 24 h (8.5 ± 3.1 nmol/gr tissue *vs* 14.7 ± 2.1 nmol/gr tissue, $P = 0.224$) (Figure 2).

Table 1 Pleural effusion volume and LW/BW ratios in different groups (mean ± SD)

Groups	Sham	ANP1	ANP2	ANP3	EPO1	EPO2	EPO3
Pleural effusion (mL)	0.18 ± 0.08	1.62 ± 1.08	1.5 ± 0.33	1.97 ± 0.39	0.45 ± 0.37	0.48 ± 0.38	0.85 ± 0.13
LW/BW ratio	0.003 ± 0.0004	0.006 ± 0.0022	0.007 ± 0.0016	0.008 ± 0.0019	0.004 ± 0.0008	0.005 ± 0.0009	0.005 ± 0.0011

Table 2 Serum amylase, IL-6, IL-2 and tissue MDA levels in different groups (mean ± SD)

Groups	Sham	ANP1	ANP2	ANP3	EPO1	EPO2	EPO3
Amylase (U/L)	534 ± 124	3502 ± 1830	3759 ± 1505	5056 ± 1872	1523 ± 514	2317 ± 311	735 ± 454
IL-6 (pg/mL)	4.7 ± 2.01	24.4 ± 3.26	27.7 ± 3.74	33.2 ± 2.1	12.2 ± 2.15	12.8 ± 1.89	13.8 ± 3.24
IL-2 (pg/mL)	7.4 ± 2.88	7.7 ± 3.17	9.3 ± 2.74	7.2 ± 3.1	6.9 ± 2.04	7.7 ± 2.93	10.6 ± 3.9
MDA (nmol/gr tissue)	8.5 ± 3.1	45.9 ± 6.8	49.3 ± 9.5	58.8 ± 9	14.7 ± 2.1	21.2 ± 2.7	30.4 ± 2.1

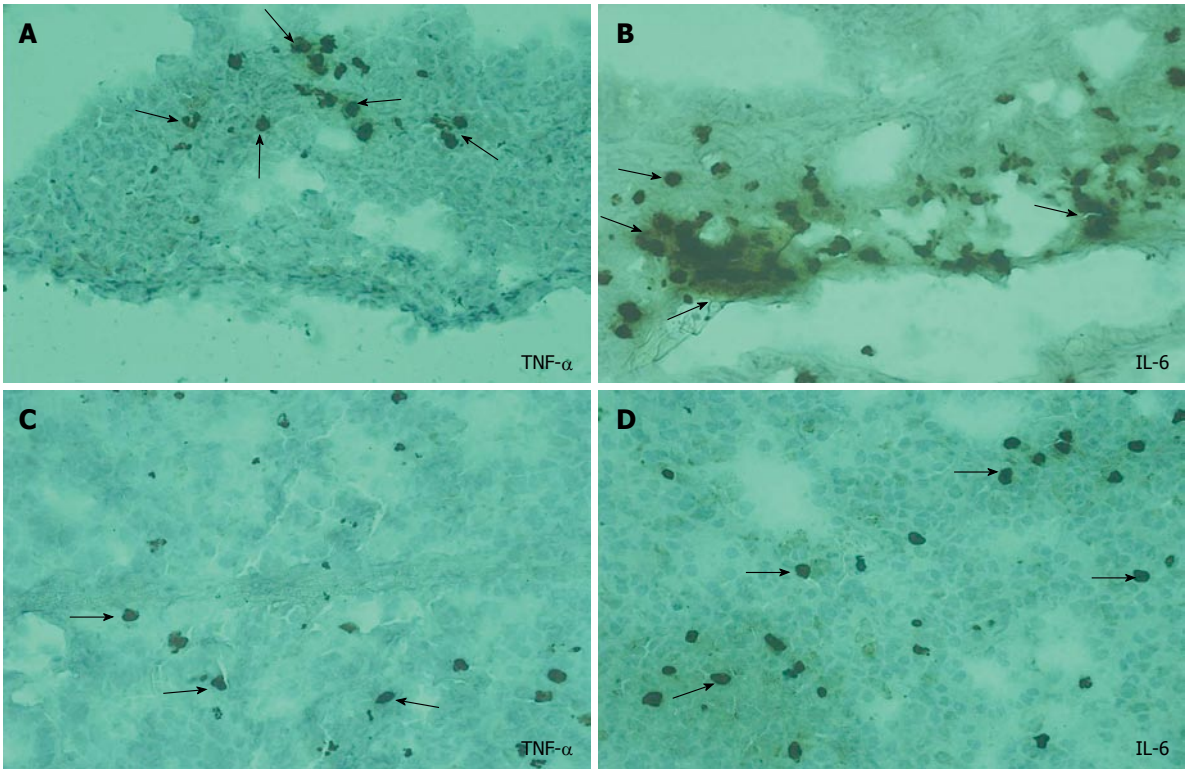

Figure 3 Light microscopic view of immunohistochemical staining for intracellular accumulations of TNF-α and IL-6 in the lung sections of ANP groups (**A** and **B**) and EPO groups (**C** and **D**) at 72 h. Arrows indicate the significantly positive staining in ANP groups (**A** and **B**) and less intensive immnunohistochemical staining in EPO groups (**C** and **D**).

Immunohistochemical screening: The intracellular accumulation of TNF-α and IL-6 was evident in the lung tissues of ANP groups (Figure 3A and B) when compared to EPO groups, particularly at 72 h (Figure 3C and D). No significant difference in IL-2 accumulation was detected among the groups.

Immune-fluorescent screening of ox-LDL: As we did not observe any positive immunofluorescent staining either in pancreas or in lung tissue of sham and EPO groups, a significant positive staining for ox-LDL was determined in ANP groups, which became much evident at 72 h (Figure 4A and B).

Histopathologic analysis

Alveolar distention and collapse: Alveolar distention and collapse were significantly intense in ANP groups at 24 h, 48 h and 72 h when compared to EPO groups ($P < 0.0001$). The alveolar distention and collapse scores (mean ± SD) for ANP and EPO groups calculated at 24 h, 48 h and 72 h were 0.85 ± 0.69, 1.57 ± 0.78, and 1.71 ± 0.75 *vs* 0.71 ± 0.75, 1 ± 0.57 and 0.42 ± 0.53 respectively. Only ANP groups demonstrated a significant difference at 72 h in comparison with sham (1.71 ± 0.75 *vs* 0.4 ± 0.54, $P = 0.03$) (Figure 5A).

Alveolar edema index: Alveolar edema index was

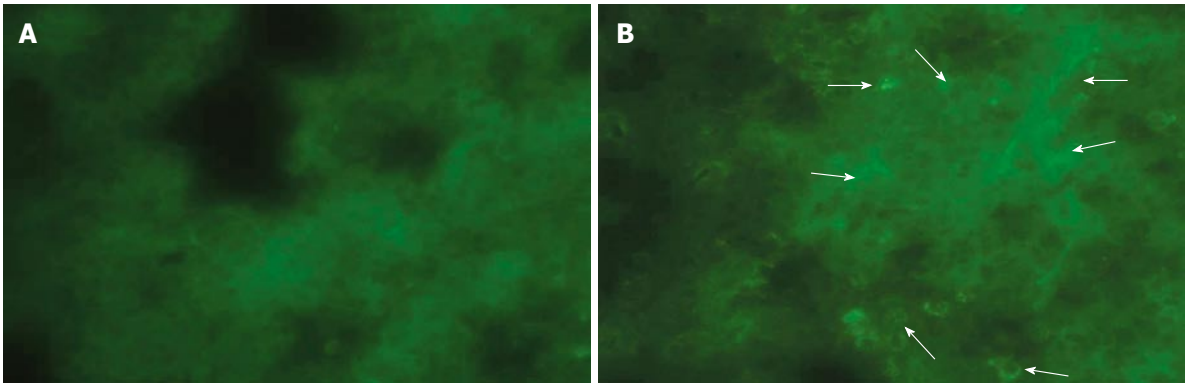

Figure 4 Lung tissue sections from EPO groups and ANP groups showing no fluorescent staining (**A**) and positive fluorescent staining (**B**) at 72 h. Arrows indicate the accumulation areas of ox-LDL in the lung.

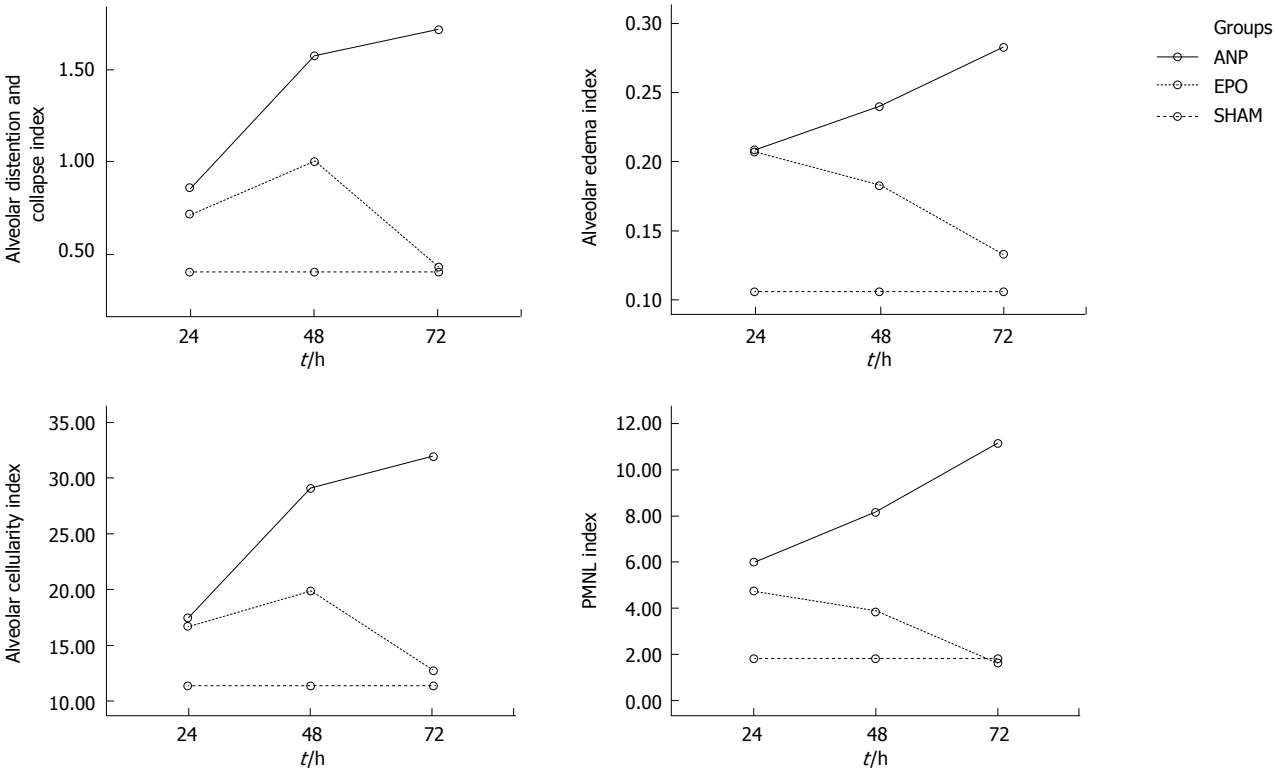

Figure 5 Mean values of alveolar distension and collapse index (**A**), alveolar edema index (percentage of the alveolar lumen filled with edema) (**B**), alveolar cellularity index (**C**) and polymorphonuclear cell index (**D**) obtained in the seven groups.

significantly different both in ANP groups and in EPO groups depending on the time course ($P = 0.002$). Alveolar edema was more intense in ANP groups at 48 h and 72 h, when compared to EPO groups (0.24 ± 0.05 *vs* 0.18 ± 0.03, $P < 0.05$ and 0.28 ± 0.03 *vs* 0.13 ± 0.04, $P < 0.01$). Moreover, at 72 h the mean alveolar edema index determined was the highest in ANP groups and the lowest in EPO groups (0.28 ± 0.03 *vs* 0.13 ± 0.04). In comparison with sham group, ANP groups had a significantly increased mean alveolar edema index at 24 h, 48 h and 72 h (0.1 ± 0.02 *vs* 0.2 ± 0.08, $P = 0.019$; 0.1 ± 0.02 *vs* 0.24 ± 0.05, $P = 0.001$; and 0.1 ± 0.02 *vs* 0.28 ± 0.03, $P = 0.0001$; respectively). On the other hand, no statistically significant difference was detected between sham group and EPO groups at 48 h and 72 h (0.1 ± 0.02 *vs* 0.18 ± 0.03, $P = 0.149$ and 0.1 ± 0.02 *vs* 0.13 ± 0.04, $P = 0.968$), which might propose that EPO treatment could decrease alveolar edema index at 48 h and 72 h (Figure 5B).

Alveolar cellularity index: Alveolar cellularity index was significantly different in either ANP groups or in EPO groups depending on the time course ($P = 0.011$). There was no significant difference in alveolar cellularity index between ANP and EPO groups at 24 h (17.42 ± 9.16 *vs* 16.71 ± 8.61, $P > 0.05$), whereas the mean value for ANP groups was significantly increased at 48 h and 72 h (29.14 ± 8.39 *vs* 19.85 ± 5.89, $P < 0.05$ and 32 ± 6.42 *vs* 12.71 ± 7.11, $P < 0.01$). Additionally, the mean alveolar cellularity index at 72 h was the highest in ANP groups and the lowest in EPO groups (32 ± 6.42 *vs* 12.71 ± 7.11).

Table 3 Histopathological index scores of lung injury (mean ± SD)

Groups	Sham	ANP1	ANP2	ANP3	EPO1	EPO2	EPO3
Alveolar distention collapse	0.4 ± 0.54	0.85 ± 0.69	1.57 ± 0.78	1.71 ± 0.75	0.71 ± 0.75	1 ± 0.57	0.42 ± 0.53
Alveolar edema index	0.1 ± 0.02	0.2 ± 0.08	0.24 ± 0.05	0.28 ± 0.03	0.2 ± 0.04	0.18 ± 0.03	0.13 ± 0.04
Alveolar cellularity index	11.4 ± 8.14	17.42 ± 9.16	29.14 ± 8.39	32 ± 6.42	16.71 ± 8.61	19.85 ± 5.89	12.71 ± 7.11
PMNL cell index	1.8 ± 1.78	6 ± 3.51	8.14 ± 3.48	11.14 ± 5.55	4.71 ± 2.36	3.85 ± 2.19	1.57 ± 1.61

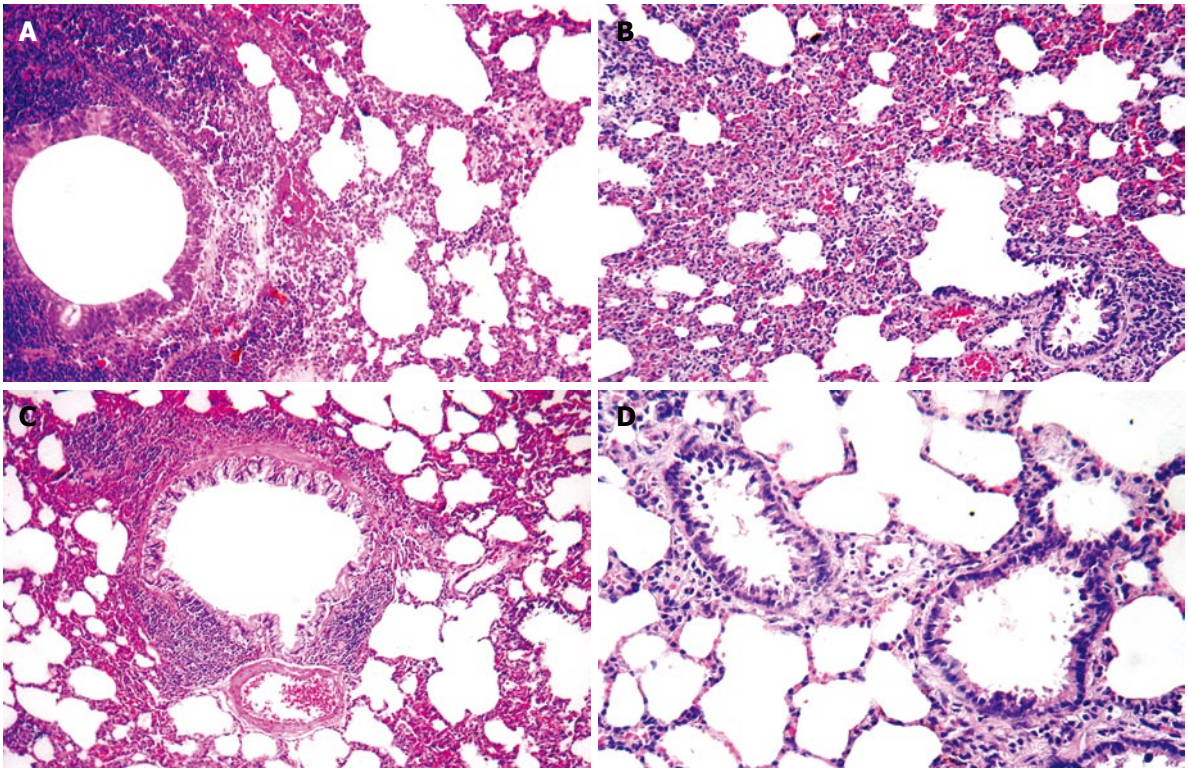

Figure 6 Light microscopy revealing significant lung injury-associated alveolar septal thickening, interstitial edema, infiltration of inflammatory cells, destruction of alveolar wall (emphysema), and microabscess formation in ANP groups at 48 h (**A**) and 72 h (**B**), and attenuation of inflammatory reaction, edema and emphysema in EPO groups at 48 h (**C**) and 72 h (**D**).

Compared with sham group, alveolar cellularity index was significantly increased in only ANP groups at 48 h and 72 h (11.4 ± 8.14 *vs* 29.14 ± 8.39, $P = 0.006$ and 11.4 ± 8.14 *vs* 32 ± 6.42, $P = 0.001$). This might suggest that EPO administration following ANP could decrease alveolar cellularity index at 48 h and 72 h (Figure 5C).

PMNL index: A statistically significant difference was observed in PMNL index between ANP and EPO groups with respect to the time intervals ($P = 0.009$). PMNL index was similar either in ANP groups or in EPO groups at 24 h (6 ± 3.51 *vs* 4.71 ± 2.36, $P > 0.05$). However, EPO treatment significantly decreased the mean PMNL index at 48 h and 72 h (8.14 ± 3.48 *vs* 3.85 ± 2.19, $P < 0.05$ and 11.14 ± 5.55 *vs* 1.57 ± 1.61, $P < 0.01$). The mean ± SD value at 72 h was the greatest in ANP groups and the lowest in EPO groups (11.14 ± 5.55 *vs* 1.57 ± 1.61). There was no statistically significant difference in PMNL index at 24 h, 48 h and 72 h between ANP and EPO groups (1.8 ± 1.78 *vs* 4.71 ± 2.36, $P = 0.315$; 1.8 ± 1.78 *vs* 3.85 ± 2.19, $P = 0.930$; and 1.8 ±1.78 *vs* 1.57 ± 1.61, $P = 1.00$, respectively), whereas ANP induction resulted in an increased PMNL index at 48 h and 72 h (1.8 ± 1.78 *vs* 8.14 ± 3.48, $P = 0.028$ and 1.8 ± 1.78 *vs* 11.14 ± 5.55, $P = 0.0001$, respectively) but not at 24 h (1.8 ± 1.78 *vs* 6 ± 3.51, $P = 0.725$). This might be explained as EPO administration could decrease PMNL index at 48 h and 72 h (Figure 5D). The histopathological indexes of lung injury (mean ± SD) are listed in Table 3. The representative light microscopic views of lung injury at 48 h and 72 h, and inecrotizing pancreatitis with severe fatty necrosis in ANP and EPO groups are shown in Figures 6 and 7. According to the above-mentioned criteria, it might be speculated that EPO administration could alleviate pulmonary injury by decreasing alveolar edema, alveolar cellularity and PMNL indexes at 48 h and 72 h following taurocolic acid-induced pancreatitis. The effect of EPO on alveolar distention and collapse was restricted at 72 h.

DISCUSSION

ANP is an inflammatory disorder with various systemic

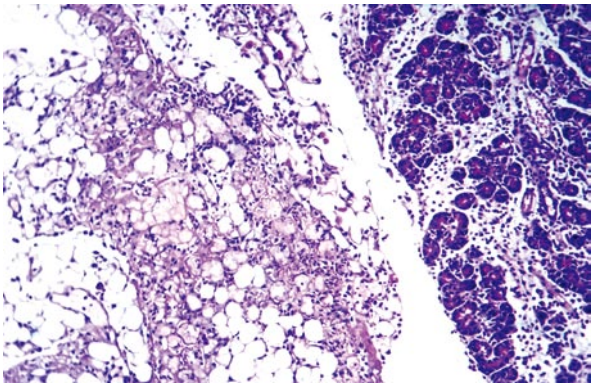

Figure 7 Light microscopic view of pancreatitis with severe fatty necrosis.

complications. ALI and ARDS are the most dreadful complications of ANP and impending catastrophe which is difficult to deal with clinically. Various medications directed at key stages of the pathophysiology are not clinically efficacious as indicated in the preceding experimental trials[23]. Therefore, therapies for preventing or reversing lung injury would be ideal for the treatment of AP[1,2,21,24-26].

Randomized studies of AP in the clinical setting do have limitations. In this regard, reliable AP animal models are of paramount importance. Taurocholate infusion model is a well-established ANP rat model that induces multiple organ failure involving the lung[27]. Moreover, Milani *et al*[28] found that mechanical and morphologic alterations in pancreatitis-associated pulmonary injury in rats are similar to those observed in humans.

The pathophysiology of ALI/ARDS and most of other pulmonary complications is multifactorial in ANP. The major pathway is the induction of a strong inflammatory response both in experimental models and in patients[29]. Regardless of the priming process, the disease progression can be viewed as three phases in continuum: local inflammation of the pancreas (a generalized inflammation stage and SIRS), and the final stage of multiorgan dysfunction[24,25]. The first sign of MODS is often the impaired lung function that manifests itself clinically as ARDS[1,2,24,25,30]. SIRS is one of the crucial reasons for pancreatitis-associated lung injury and PMNL plays a central role with various inflammatory cytokines and reactive oxygen species (ROS)[2,29,31]. Many researchers have focused their efforts on preventing AP-induced lung injury by pharmacologic interventions. Attractively, recent works have discovered the potential role of EPO as a multifunctional endogenous mediator offering cytoprotective effect against injury in various tissues including lung[16-19]. In multiple species including humans, many tissues injured by ischemia, mechanical trauma, excitotoxins, and other stressors are significantly improved by administration of EPO following injury[32]. The presence of a therapeutic window dictates specific time constraints for efficacious administration of exogenous EPO as a cytoprotectant[5]. According to this hypothesis we administered EPO immediately following the induction of pancreatitis and evaluated its effect in three different time courses.

The principle mechanism by which EPO confers tissue protection involves the modulation of cellular apoptosis. EPO inhibits the apoptotic mechanisms of injury, including preservation of cellular membrane asymmetry to prevent inflammation, can therefore be regarded as a general tissue-protective cytokine[11,12,14]. Agents that can prevent apoptosis can be effective long after the occurrence of injury[5]. This phenomenon might describe the long protective effect of EPO on lung injury in our study, particularly at 48 h and 72 h. We would also like to emphasize that, in patients with a severe attack, the effects of distant organ damage including lung injury, are often not fully established and become apparent only over the following 48 h. There is thus a therapeutic window between hospital presentation and development of distant organ dysfunction. As an obvious time window existed in this process, therapeutic approach should focus on it during this period. From this assumption, the animals were sacrificed on postoperative hours 24, 48 and 72 for histopathological and biochemical evaluations in our study.

Another crucial determinant for observing the cytoprotective effect of EPO is the serum concentration. The serum concentration of EPO required for tissue protection is higher than that required for erythropoiesis. Preclinical data suggest that the minimum therapeutic level needed for protection against tissue injury appears to be 300-500 mIU/kg body weight (intravenously or intraperitoneally) for the organs to be adequately investigated. EPO administration (100-1000 U/kg body weight) achieves possible systemic protective effects whereas high doses of EPO (3000-5000 U/kg body weight) are necessary for cardioprotection and neuroprotectin[33]. According to this, we administered EPO at the dose of 1000 mIU/kg body weight to observe the cytoprotective effects.

EPO plays a dual role in vascular protection by preserving endothelial cell integrity[6,8,9], thus playing a role in maintaining the integrity of microvasculature[11]. One of the major factors for the development of alveolar edema in ANP is the increased microvascular permeability. The experimental protocol we performed let us to measure the amount of edema within alveoli. We used alveolar edema index and alveolar distension and collapse index as markers of ALI according to the previous observations suggesting that histologic evidence of pulmonary tissue injury can appear before the development of clinically relevant respiratory mechanical changes[22]. We prefer three different time courses, since pulmonary injury indexes are quite intense in taurocolic acid induced acute pancreatitis on d 1 and 3, some of which persist through d 8[22]. In the present study, pulmonary edema, alveolar cellularity index and PMNL index (pulmonary injury index) were significantly reduced in EPO groups at 48 h and 72 h, suggesting that EPO can preserve endothelial cell integrity.

Oxidative stress has been implicated as a crucial landmark by increasing endothelial permeability in ARDS[1]. ROS scavengers possess protective effect against local acute pancreatitis-associated with lung injury[21,34,35]. In addition to other effects, EPO has been demonstrated in various tissues to be an antioxidant as it can decrease

the plasma iron concentration and increase the ability of plasma to inhibit lipid peroxidation[36,37]. In the present study, we determined the tissue levels of thiobarbituric acid reactant MDA, which is considered a good indicator of lipid peroxidation, and found a significant decrease in EPO group when compared to ANP groups in all three time courses. This might be attributed to the antioxidant effect of EPO. Furthermore, the tissue damage induced by ANP was associated with a significant ox-LDL accumulation either in pancreas samples or in lung tissue specimens. Ox-LDL is an early product of lipid peroxidation and ox-LDL accumulation in pancreatitis is associated with lung injury.

At present, the role of inflammatory mediators in the pathogenesis of ARDS has become a hot issue in the research field. EPO has been demonstrated to prevent cellular inflammation by inhibiting several proinflammatory cytokines, such as IL-6, TNF-α, and monocyte chemoattractant protein[7,38]. Attractively, these effects of EPO can be mediated by both hormonal and paracrine modalities[38]. There is mounting evidence that proinflammatory cytokines are the agents behind the systemic complications of AP[1,2]. It was reported that systemic inflammation plays a role in development of ALI triggered by pancreatitis[30]. The critical players of this process include proinflammatory cytokines including IL-1β, TNF-α, IL-6, IL-8, and platelet activating factor (PAF)[29]. Among these, the serum and/or tissue levels of TNF-α, IL-2 and IL-6 were analyzed in this study. Regardless of the model of acute pancreatitis, inhibition of the potent cytokine TNF-α might decrease organ injury and improve survival[39]. The tissue levels of TNF-α in the lungs were analyzed with immunohistochemical staining. Since no quantitative analysis was possible unavailable techniques, we evaluated this parameter not statistically but morphologically. IL-6 is another proinflammatory cytokine, and its high circulating level has been shown to be an excellent predictor of the severity of ARDS with different etiologies, including AP[40]. Moreover, IL-6 has been proposed to be one of the best prognostic parameters for pulmonary failure in human AP[30,41]. Mayer *et al*[41] have confirmed the important role of soluble IL-2 receptors (a lymphocyte activation marker), as a marker for severe AP, especially when complicated by lung or kidney failure or sepsis during lethal course of the disease[41]. In the present study, pulmonary injury in ANP groups was characterized by the increased serum or tissue IL-6 and TNF-α level. EPO treatment significantly decreased IL-6 and TNF-α level which might be due to the antiinflammatory properties of its molecule. However, we did not determine a statistically significant difference in the IL-2 level among the groups. This result might reflect the ineffectiveness of EPO on lymphocyte activity.

In conclusion, EPO administration plays a crucial role in preventing histological changes of ALI induced by experimental ANP. Moreover, it can significantly reduce the circulating and tissue levels of proinflammatory cytokines which have been considered the key factors for ALI. Additionally, oxidative stress markers are decreased particularly at 72 h following the induction of pancreatitis that might be attributed to the long-lasting antioxidant effect of EPO. All these findings show that EPO can attenuate ANP-induced lung injury by inhibiting PMNL accumulation, decreasing the circulating levels of proinflammatory cytokines, preserving microvascular endothelial cell integrity and reducing oxidative stress-associated lipid peroxidation. Years of clinical application in patients with anemia and chronic renal disease indicate that EPO is safe and well tolerated and can act as an ideal cytoprotective agent[7,42,43]. Nevertheless, the issue which should also be taken into consideration is that EPO is not an absolutely innocent agent with subsequent clinical toxic effects. Therefore, it would be of value to investigate its pharmacodynamics, pharmacokinetics, side-effects, administration routes and doses before used as a potential candidate for the treatment of ANP-associated ALI in routine clinical practice. In other words, this is a preliminary study and more experiments are necessary for the efficacy and potentially cytoprotective mechanisms of EPO action.

COMMENTS

Background

Pulmonary complication is the major cause for mortality in acute necrotizing pancreatitis (ANP). Since no absolutely effective treatment is available at present, therapies for preventing or reversing lung injury would be ideal for the treatment of AP.

Research frontiers

Erythropoietin (EPO) has long been known as a glycoprotein hormone that regulates erythropoiesis in mammals. Beyond its hematopoietic properties, EPO modulates a broad array of vital cellular processes including progenitor stem cell development, cellular integrity, and angiogenesis. EPO has recently been demonstrated to play a role in prolonging cell survival by acting as an antiapopitotic agent. EPO inhibits the apoptotic mechanisms of injury including preservation of cellular membrane asymmetry to prevent inflammation, and can therefore be regarded as a general tissue-protective cytokine. Additionally, experimental evidence supports a vigorous cytoprotective effect of EPO, which is now considered to have applicability in a variety of disorders, such as cerebral ischemia, myocardial infarction, and chronic congestive heart failure.

Related publications

The present study was an experimental study addressing the beneficial effects of EPO on lung injury. We cited several articles from other investigators reporting researches of EPO action on various tissues including lungs.

Innovations and breakthroughs

Recent works have discovered the potential role of EPO as a multifunctional endogenous mediator offering cytoprotective effect against injury in various tissues including the lungs. Pretreatment with EPO appears to attenuate ischemia-reperfusion-induced lung injury and hyperoxic lung injury in neonatal rats. From this point of view we evaluated the potantial protecting effects of EPO against acute lung injury in a rat model of ANP. Our data show that EPO administration can alleviate pulmonary injury parameters in experimental pancreatitis.

Applications

The impending catastrophe in ANP is generally preceded by acute lung injury. Despite improved understanding of the pathogenesis of ARDS, pharmacological modalities are ineffective in decreasing its mortality. None of the randomized clinical trials using novel therapeutic agents has demonstrated an improvement in patient outcome. The verification of cytoprotective effects of EPO on acute lung injury in a model of experimental pancreatitis might shed some valuable light on the novel effective therapeutic interventions.

Terminology

Erythropoietin (EPO), a 30.4-kDa glycoprotein and a member of the type I

cytokine superfamily, was first introduced as a hormone that regulates erythroid progenitors within the bone marrow to mature into erythrocytes, through binding to its specific cell surface receptors. Acute necrotizing pancreatitis (ANP) is a life-threatening necroinflammatory disease of pancreas with significant morbidity and mortality rates. Acute lung injury (ALI) is one of the most dreadful complications of AP which might be described as the continuum of pathological responses to pulmonary parenchymal injury. Acute respiratory distress syndrome (ARDS) is a severe form of ALI and acute pulmonary inflammation syndrome and resultant increased capillary endothelial permeability with clinical features of severe dyspnea and extreme hypoxemia refractory to a high inspired oxygen concentration.

Peer review

This is a well-designed and interesting study about the beneficial effects of EPO on lung injury in an experimental model of ANP. Since this is a preliminary study as discussed by the authors, more comprehensive experiments should be carried out to reveal the underlying cellular mechanisms of EPO's cytoprotective action against lung injury.

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BASIC RESEARCH

Carbon liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice

Bing-Wei Sun, Qin Jin, Yan Sun, Zhi-Wei Sun, Xi Chen, Zhao-Yong Chen, Gediminas Cepinskas

Bing-Wei Sun, Qin Jin, Yan Sun, Zhi-Wei Sun, Xi Chen, Zhao-Yong Chen, Department of Burns and Plastic Surgery, Affiliated Hospital, Jiangsu University, Zhenjiang 212001, Jiangsu Province, China
Gediminas Cepinskas, Centre for Critical Illness Research, Lawson Health Research Institute, 800 Commissioners Rd. E., London, Ontario, N6A 4G4, Canada
Correspondence to: Dr. Bing-Wei Sun, Department of Burns and Plastic Surgery, Affiliated Hospital, Jiangsu University, 438 Jiefang Rd, Zhenjiang 212001, Jiangsu Province, China. sunbinwe@hotmail.com
Telephone: +86-511-85026183 Fax: +86-511-5029089
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Abstract

AIM: To determine whether Carbon (CO) liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice.

METHODS: Thirty-six mice were assigned to four groups. Mice in the sham group (n = 9) were underwent to sham thermal injury; mice in the burn group (n = 9) received 15% total body surface area full-thickness thermal injury; mice in the burn + CORM-2 group (n = 9) were underwent to the same thermal injury with immediate administration of tricarbonyldichlororut henium (Ⅱ) dimer CORM-2 (8 mg/kg, i.v.); and mice in the burn+DMSO group (n = 9) were underwent to the same thermal injury with immediate administration of 160 μL bolus injection of 0.5% DMSO/saline. Histological alterations and granulocyte infiltration of the small intestine were assessed. Polymorphonuclear neutrophil (PMN) accumulation (myeloperoxidase assay) was assessed in mice mid-ileum. Activation of nuclear factor (NF)-κB, expression levels of intercellular adhesion molecule-1 (ICAM-1) and inducible heme oxygenase in mid-ileum were assessed.

RESULTS: Treatment of thermally injured mice with CORM-2 attenuated PMN accumulation and prevented activation of NF-κB in the small intestine. This was accompanied by a decrease in the expression of ICAM-1. In parallel, burn-induced granulocyte infiltration in mid-ileum was markedly decreased in the burn mice treated with CORM-2.

CONCLUSION: CORM-released CO attenuates leukocyte infiltration in the small intestine of thermally injured mice by interfering with NF-κB activation and protein expression of ICAM-1, and therefore suppressing the pro-adhesive phenotype of endothelial cells.

Key words: Leukocyte infiltration; Carbon monoxide; Thermal injury; Small intestine

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INTRODUCTION

Systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF) still continue to be leading causes of morbidity and mortality in severe burn patients[1,2]. The intestine is considered to be the critical organ in the development of organ dysfunction in trauma, burns, and intensive care unit patients[3]. Thermal injury is accompanied by complex events that exert deleterious effects on various organs, such as the small intestine, distant from the original burn wound. Following thermal injury, the small intestine is subjected to ischemia, and consequently, especially during burn resuscitation, reperfusion injury occurs[4]. Intestinal ischemia-reperfusion results in organ injury through both tissue hypoxia and reperfusion phenomena mediated by neutrophils[5,6]. A variety of cytokines are released into the microcirculation by neutrophils, endothelial cells and monocytes during phases of hypoxia and reperfusion[7,8]. Although the pathophysiological basis of organ damage remains unclear, there is increasing evidence that leukocyte infiltration into intestinal tissue plays an important role in bacterial or endotoxin translocation and development of SIRS after thermal injury[9-12].

A lot of evidence indicates that endogenous Carbon (CO), a by-product of inducible heme oxygenase (HO-1), modulates inflammation. In addition, some experiments have determined that the administration of exogenous CO inhibits lipopolysaccharide (LPS)-induced production

of cytokines both *in vivo* and *in vitro*, and consequently exhibits an important cytoprotective function and anti-inflammatory properties that are beneficial for the resolution of acute inflammation[13-15].

Recently, transitional metal carbonyls have been identified as potential CO-releasing molecules (CORMs), with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs[16]. CORMs have been shown to act pharmacologically in rat aorta and cardiac tissue in which liberation of CO induced vasorelaxant effects[17-20] and decreased myocardial ischemia-reperfusion injury[21,22], respectively. Our previous studies[23,24] have shown that burn-induced overexpression of adhesion molecules [such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1] on endothelial cells and leukocytes may contribute to liver and lung tissue injury, subsequently leading to multiple organ dysfunction syndrome (MODS). We also confirmed that CORM-released CO attenuated leukocyte sequestration in the liver and lungs of burned mice by interfering with NF-κB activation and protein expression of ICAM-1, therefore suppressing the pro-adhesive phenotype of endothelial cells. However, it is still unknown if CORM-released CO can exert its anti-inflammatory and protective effects in the small intestine. Based on these preliminary observations, in this study we employed tricarbonyldichlororuthenium (Ⅱ) dimer (CORM-2), one of the novel group of CORMs, to determine whether it attenuated leukocyte infiltration to the intestinal tissue of thermally injured mice.

MATERIALS AND METHODS

Materials

CORM-2 was obtained from Sigma-Aldrich and was solubilized in DMSO to obtain a 10 mmol/L stock solution. Polyclonal or monoclonal antibodies against ICAM-1 and HO-1 were purchased from Santa Cruz Biotechnology. All other chemicals were reagent grade and obtained from Sigma unless otherwise stated.

Animals and burn protocol

C57BL/6 mice (36 male; body weight 20 ± 2 g) were fed a standard laboratory diet and water *ad libitum*. Mice were assigned to four groups. Mice in the sham group (n = 9) were underwent to sham thermal injury; mice in the burn group (n = 9) received 15% total body surface area (TBSA) full-thickness thermal injury; mice in the burn + DMSO group (n = 9) were underwent to the same thermal injury with immediate administration of 160 μL bolus injection of 0.5% DMSO/saline; and mice in the burn + CORM-2 group (n = 9) were underwent to the same thermal injury with immediate administration of CORM-2 (8 mg/kg, i.v.). The experimental protocol was approved by The Council on Animal Care at Jiangsu University for the protection and welfare of animals. Under anesthesia with spontaneous inhalation of isoflurane/N_2O (Abbott Laboratories, Mississauga, ON, Canada) in a 60% oxygen/40% nitrogen mixture, the dorsum of each mouse was shaved and the animal was subjected to 15% TBSA full-thickness thermal injury as previously described[25,26]. Sham animals were immersed in a water bath at room temperature. All animals were resuscitated with 1.5 mL saline immediately after thermal (or sham) injury. No wound care was required for the burn wounds. This burn method achieves a histologically proven, full-thickness scald injury[27,28]. The animals were sacrificed at 24 h after experimental manipulation.

Table 1 Histological scoring system for ileum and jejunum sections stained with hematoxylin/eosin

	Granulocyte infiltration			Hydropic degeneration		
	0	1	2	0	1	2
Ileum	No	Moderate	Intense	No	Moderate	Intense
Jejunum	No	Moderate	Intense	No	Moderate	Intense

Ileum histologic studies

The mid-ileum specimens harvested from different groups of animals were immersed in 4% formaldehyde solution at 24 h after thermal injury. The tissue was embedded in paraffin wax, serially sectioned, and stained with hematoxylin-eosin. Ileal morphologic characteristics were evaluated by light microscopy. Ileum tissue was evaluated for density of granulocytes and degree of hydropic degeneration. Tissues were evaluated in a semi-quantitative manner by two experienced independent examiners that were blinded to the experimental groups (Table 1). A scoring system was used for each item using 0 up to 2 points for the different states of organ damage (with 2 being most granulocytes, edema and degeneration; Table 1). Afterwards, the mean ± SEM of each item was calculated.

Preparation of intestinal homogenates

Immediately after withdrawing blood, the intestine was exposed. Leaving approximately the first 5-cm-long proximal segment of the intestine, 3-cm-long segments of jejunum and ileum were removed, cleaned, and snap-frozen in liquid nitrogen. The samples were stored at -70℃. Equal weights (100 mg wet weight) of intestine from various groups were suspended in 1 mL PBS and sonicated (30 cycles, twice, for 30 s) on ice[29]. Homogenates were cleared by centrifuging at 12000 r/min at 4℃, and the supernatants were stored at -70℃. Protein levels in the homogenates were determined using the Bio-Rad (Hercules, CA, USA) assay kit.

Myeloperoxidase (MPO) activity

MPO activity was measured in ileum tissue using a procedure similar to that documented by Hillegas *et al*[30,31]. Tissue samples were homogenized in 50 mmol/L potassium phosphate buffer (PB) (pH 6.0), and centrifuged at 10000 × g (10 min); pellets were suspended in 50 mmol/L PB containing 0.5% hexadecyltrimethylammonium bromide. After sonication, the samples were centrifuged at 10000 × g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mmol/L PB, o-dianisidine, and 20 mmol/L H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO

Table 2 Histological scoring (score in Table 1) of ileum and jejunum tissue stained with hematoxylin/eosin 24 h after thermal injury

	Granulocyte infiltration			Hydropic degeneration		
	Sham	Burn	Burn + CORM	Sham	Burn	Burn + CORM
Ileum	0.60 ± 0.1	1.92 ± 0.4[a]	0.88 ± 0.2[c]	0	1.8 ± 0.2	1.7 ± 0.1
Jejunum	0.54 ± 0.1	1.5 ± 0.2[a]	0.64 ± 0.2[c]	0	1.6 ± 0.1	1.6 ± 0.2

0, no injury; 1, moderate injury; 2, severe injury. Values are presented as mean ± SEM. [a]$P < 0.05$ *vs* sham group; [c]$P < 0.05$ *vs* burn group.

present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Measurement of ICAM-1

ICAM-1 levels in ileum tissue homogenates were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Western blot analysis

Tissues were homogenized for extraction in ice-cold mild lysis buffer, containing 1% Nonidet P-40, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.2), 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 0.2 mmol/L sodium vanadate, and 1 μg/mL aprotinin. The tissue homogenates were centrifuged at 20000 × *g* for 15 min and supernatants were collected. SDS-PAGE was performed on equivalent amounts of protein samples using precast 7% resolving/4% stacking Tris/HCl gels (Bio-Rad, Hercules, CA, USA). Separated proteins were then transferred to PVDF membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked in 5% non-fat milk in TBS buffer containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Blocked membranes were incubated with primary antibodies specific for mouse ICAM-1 and HO-1 at a concentration of 1:1000 and 1:5000, respectively, in TBST overnight at 4℃. Then, the membranes were washed and probed with horseradish-peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at room temperature. Chemiluminescence detection was performed with the Amersham enhanced chemiluminescence detection kit according to the manufacturer's instructions. To ensure a similar amount of protein in each sample, the membranes were "stripped off", reprobed with actin, developed with horseradish-peroxidase-conjugated secondary antibody, and visualized by enhanced chemiluminescence.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear protein from ileum tissue was extracted using our previously described method[32,33]. Briefly, frozen tissues were weighed, transferred to Corex tubes and homogenized in four volumes (w/v) of PBS containing 2 mmol/L phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 3000 × *g* for 10 min, and the pellet was then resuspended in 2 mL buffer A [0.3 mol/L sucrose, 5 mmol/L dithiothreitol (DTT), 5 mmol/L $MgCl_2$ 10 mmol/L Tris/HCl, 0.1% Triton X-405], and further homogenized using a Dounce homogenizer. After filtration through a 100-μm nylon mesh, the suspension was centrifuged at 1000 × *g* for 5 min at 4℃. The pellet (nuclei) was washed in buffer A (without 0.1% Triton X-405) and centrifuged (1000 × *g* for 5 min at 4℃), and then the nuclei were extracted on ice for 30 min in 60 μL buffer B containing 20 mmol/L HEPES, 0.75 mmol/L spermidine, 0.15 mmol/L spermine, 0.2 mmol/L EDTA, 2 mmol/L ethylene glycol-bis (b-aminoethyl ether)-*N, N, N', N'*-tetraacetic acid, 2 mmol/L DTT, 20% glycerol, and 1 mmol/L PMSF (4℃) in the presence of 0.4 mol/L NaCl. Finally, the samples were centrifuged for 10 min at 21000 × *g* (4℃), and the supernatants were collected and stored at -80℃ as the nuclear protein fraction.

For EMSA, 5 μg total nuclear proteins was incubated with 1.0 pmol double-stranded γ[^{32}P] ATP end-labeled oligonucleotides containing consensus binding sequences for NF-κB (sense strand 5'-AGGGACTTTCCGCTG GGGACTTTCC-3') in a binding buffer (10 mmol/L HEPES, pH 7.9, 80 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, and 10% glycerol), as described previously[34]. Samples were incubated for 30 min at room temperature and then run through a 4% non-denaturing polyacrylamide gel (0.5 × TBE buffer) at 280 V for 1 h. The gel was dried and then exposed to X-ray film (Kodak) for 4-6 h in cassettes at -80℃. Signal detection and quantification were performed by computer-assisted densitometry.

Statistical analysis

All the values are presented as mean ± SE. Statistical analysis was performed by ANOVA and Student's *t* test for the comparisons. $P < 0.05$ was considered to be statistically significant.

RESULTS

Histology

Histological analysis showed that the ileum from sham mice had the normal architecture of the intestinal epithelium and wall, while thermal injury induced severe edema and sloughing of the villous tips, as well as infiltration of inflammatory cells into the mucosa (Figure 1). Semi-quantitative analysis of histological samples of ileum and jejunum showed that granulocyte infiltration in the burned mice was significantly increased compared to that in the sham group. Administration of CORM-2 (8 mg/kg, i.v.), significantly decreased granulocyte infiltration. However, CORM-2 did not improve the hydropic degeneration induced by thermal injury in either the ileum or jejunum (Table 2).

Effect of CORM-2 on MPO activity in small intestine of thermally injured mice

To determine whether the burn-induced increase in polymorphonuclear neutrophil (PMN) accumulation in the small intestine was effectively prevented by CORM-2,

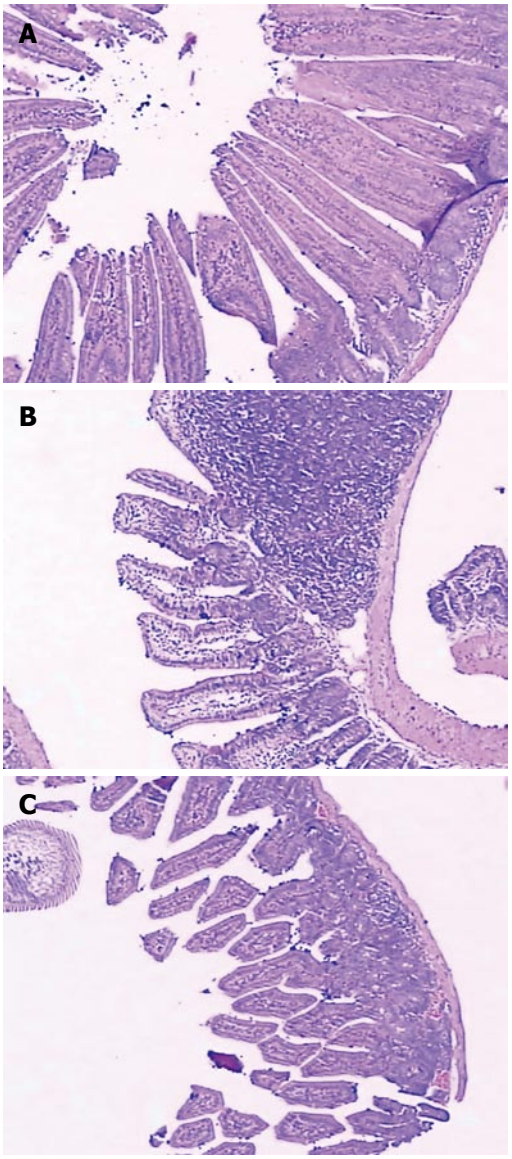

Figure 1 Effects of CORM-2 on small intestine injury in thermally injured mice. Mice were injected i.v. with CORM-2 (8 mg/kg) immediately after thermal injury. Mice in the DMSO group received a 160-μL bolus injection of 0.5% DMSO/saline. Mid-ileum sections from sham-treated mice had normal architecture of the intestinal epithelium and wall (**A**); Mid-ileum sections from thermally injured mice showed inflammatory cell infiltration through the wall, concentrated below the epithelial layer, edema of the distal portion of the villi, and necrosis of the epithelium at the villous tips (**B**); Ileum section from burned mice treated with CORM-2 (**C**) showed a significant decrease in granulocyte infiltration, while no marked improvement of hydropic degeneration. The figure is representative of at least three experiments performed on different days.

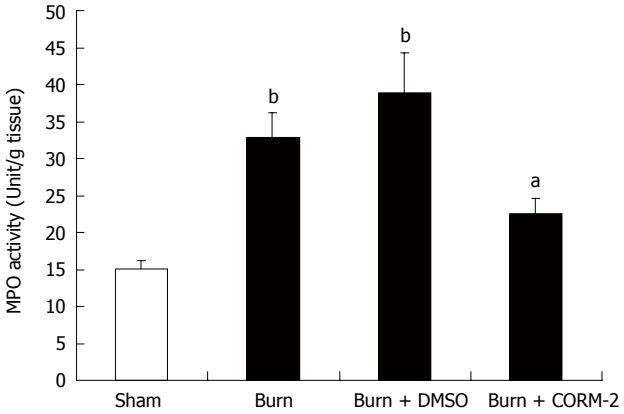

Figure 2 Effects of CORM-2 on MPO activity in the small intestine of thermally injured mice. Mice were challenged with thermal injury and treated with CORM-2 as described in Figure 1. MPO activity in the mid-ileum was assessed 24 h following thermal injury. Results are mean ± SE, $^{b}P < 0.01$ *vs* sham mice. $^{a}P < 0.05$ *vs* burned mice.

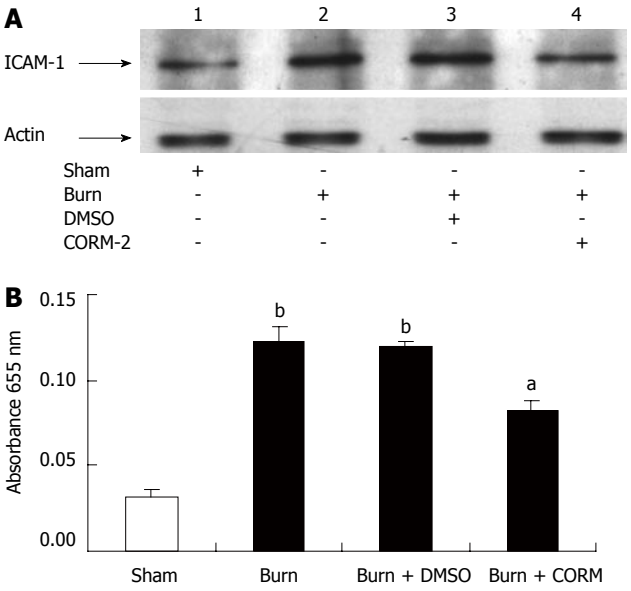

Figure 3 Effects of CORM-2 on protein expression of ICAM-1 in the ileum tissue of thermally injured mice. Mice were challenged with thermal injury and treated with CORM-2 as described in Figure 1. Protein expression of ICAM-1 was analyzed by Western blotting (**A**) and ELISA (**B**) 24 h after thermal injury. A representative experiment is shown in A. $^{b}P < 0.01$ *vs* sham-treated; $^{a}P < 0.05$ *vs* burned mice.

the activity of MPO, an enzyme in azurophilic granules of neutrophils, was assessed. Extracts of the ileum samples were examined for content of MPO 24 h after thermal injury. The mean MPO levels are shown in Figure 2. MPO activity in organs obtained from burned mice was markedly increased compared to that in the sham group ($P < 0.01$), while it was significantly decreased by treatment with CORM-2 ($P < 0.05$).

Effect of CORM-2 on expression of ICAM-1 in the small intestine of thermally injured mice

At 24 h after a 15% TBSA full-thickness thermal injury, the expression of ICAM-1 in the ileum was significantly increased compared to that in the sham-treated animals. Administration of CORM-2 (8 mg/kg, i.v.) significantly decreased expression of ICAM-1 (Figure 3).

Effect of CORM-2 on expression of HO-1 in the small intestine of thermally injured mice

At 24 h after 15% TBSA full-thickness thermal injury, the expression of HO-1 in the small intestine significantly increased compared to that in the sham-treated animals. *In vivo* administration of CORM-2 (8 mg/kg, i.v.), expression of HO-1 in the ileum tissue of burn mice was more significantly increased compared to burn group (Figure 4).

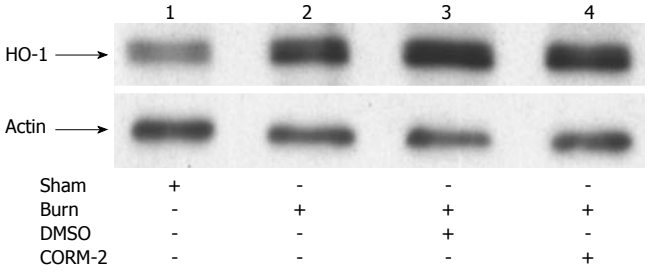

Figure 4 Effects of CORM-2 on protein expression of HO-1 in the ileum tissue of thermally injured mice. Mice were challenged with thermal injury and treated with CORM-2 as described in Figure 1. Protein expression of HO-1 was performed by Western blotting 24 h after thermal injury. A representative experiment showed that HO-1 was significantly up-regulated by thermal injury (lane 2). Expression of HO-1 in the small intestine of thermally injured mice treated with CORM-2 was more significantly increased compared to burned mice without CORM-2 (lane 4).

Effect of CORM-2 on activity of NF-κB in the small intestine of thermally injured mice

Binding of nuclear protein to the radiolabeled consensus binding sequences of NF-κB was assessed by EMSA. At 24 h after 15% TBSA full-thickness thermal injury, NF-κB activation in the ileum was markedly increased, and this was markedly inhibited by administration of CORM-2 (8 mg/kg i.v.) (Figure 5).

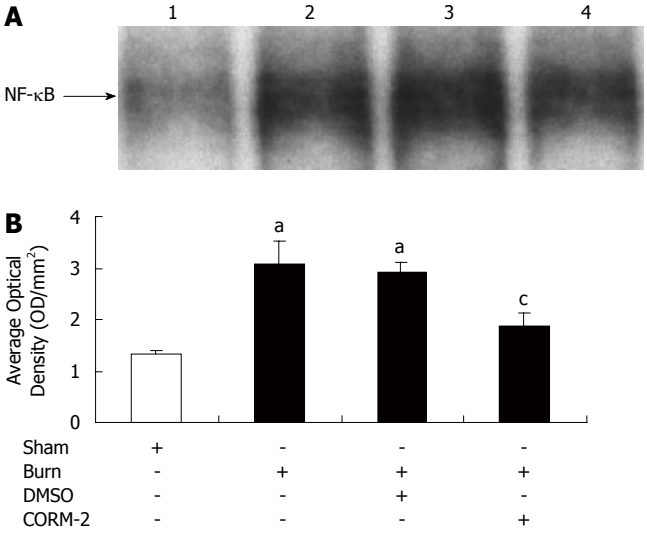

Figure 5 Effects of CORM-2 on NF-κB activation in the ileum tissue of thermally injured mice. Mice were challenged with thermal injury and treated with CORM-2 as described in Figure 1. Measurement of NF-κB activity was performed by EMSA with ^{32}P-labeled NF-κB probe and 5 μg nuclear extract from the ileum of sham, burn, burn + DMSO and burn+CORM-2 mice at 24 h after thermal injury. NF-κB activation in the ileum of thermally injured mice was markedly increased (lane 2), and this activity was inhibited by CORM-2 (lane 4). A representative experiment is shown in **A**, and quantitative results (average optical density) of three experiments are shown in **B**. $^{a}P < 0.05$ *vs* sham-treated; $^{c}P < 0.05$ *vs* burned.

DISCUSSION

Major burns alter immune function, which produces an imbalance between pro- and anti-inflammatory cytokine synthesis, and increases susceptibility to post-burn infection and sepsis[35-37]. Also, severe burns cause damage to multiple organs distant from the original burn wound, leading to MOF, a serious clinical problem. The intestine is one of the most sensitive tissues to ischemia and reperfusion induced by thermal injury. PMNs may play an important role in ischemic injury, and reperfusion of intestine is associated with accumulation of PMNs in the intestinal tissue. It has been suggested that tissue accumulation of PMNs is a key event that determines the severity of ischemia-reperfusion injury[8].

We report here that CORM-released CO exerts a protective effect against the pathological changes caused by thermal injury of the small intestine. Importantly, this exogenous CO showed effective inhibition of activation of NF-κB and expression of ICAM-1. Thus, we propose that CORM-2 contributes to the attenuation of leukocyte infiltration to the intestinal tissue after burn challenge. What is, then, the mechanism by which attenuation of PMN infiltration to the intestine is caused by thermal injury?

Many experimental studies have highlighted the specific and independent role of exogenous CO (i.e. CO inhalation) in the modulation of inflammation[38,39]. Recently some new metal carbonyl-based compounds (CORMs) that have the ability to release CO in biological systems have been identified and synthesized. The vasoactive, antihypertensive and anti-rejection effects of CORMs have been demonstrated to be due to the CO liberated by the compounds. CORM-2, a DMSO-soluble CORM, also has exhibited anti-inflammatory actions in an *in vitro* model of LPS-stimulated murine macrophages[40].

MPO is an enzyme that is found predominantly in the azurophilic granules of PMNs. Tissue MPO activity is frequently utilized to estimate tissue PMN accumulation in inflamed tissues, and correlates significantly with the number of PMNs determined histochemically in tissues[41]. In the present study, we found that intestinal MPO activity was markedly elevated after thermal injury, and administration of CORM-2 led to significant down-regulation of MPO activity. This indicates that CORM-2 effectively prevents PMN chemotaxis and infiltration in the small intestine after thermal injury, which consequently decreases the production of oxidants and reduces tissue oxidative injury, which contributes to MODS. In parallel, histological analysis in this study indicated that mid-ileum sections from thermally injured mice showed inflammatory cell infiltration through the wall, concentrated below the epithelial layer, edema of the distal portion of the villi, and necrosis of the epithelium at the villous tips. On the contrary, ileum sections from mice treated with CORM-2 showed a significant decrease in leukocyte infiltration.

PMN-endothelial cell interactions are supposed to play a central role in the pathogenesis of intestinal barrier failure following thermal injury and ischemia-reperfusion[42]. The presence of ICAM-1, which mediates leukocyte adhesion, correlates with infiltration of leukocytes into inflammatory lesions[43,44]. It seems to be the initial marker of inflammatory reactions and is involved in the acute inflammatory reaction following burns[45]. ICAM-1 activates leukocytes and endothelial cells, which in turn, prompt the release of various inflammatory mediators. This may result in SIRS, acute respiratory distress syndrome, and MODS, which may develop further into progressive MOF and death[46-48]. The present

results showed that at 24 h post-burn, the expression of ICAM-1 in intestinal tissue was markedly up-regulated. CORM-2 was able to inhibit the up-regulation of ICAM-1 induced by thermal injury. Our findings strongly indicate that CORM-2 appears to inhibit leukocyte activation and adhesion, and consequently, might effectively decrease the inflammatory response in the small intestine induced by burns.

HO is a rate-limiting enzyme that is responsible for the catabolism of heme into bilirubin, free iron, and CO. Three HO isoforms have been identified: HO-2 and HO-3 isoforms are believed to be constitutive and physiologically expressed, whereas HO-1 isoform is a stress-responsive protein that is induced by various stimuli. The adaptive response of HO-1 to various stimuli suggests that it may play an important role in protection against the inflammatory response and oxidative injury[49]. Other studies have shown that up-regulation of endogenous HO-1 ameliorates inflammatory responses and/or tissue damage[50]. In this study, we found that HO-1 was significantly up-regulated by thermal injury. Interestingly, the expression of HO-1 in the small intestine of thermally injured mice treated with CORM-2 was more significantly increased compared to burned mice without CORM-2 (Figure 4). This result indicates that not only major burn injury might significantly induce the expression of HO-1, but also the increase in HO-1 expression can be further enhanced by the administration of CORM-2. Through the by-product (CO and/or biliverdin), the potent cytoprotective and anti-inflammatory functions were ultimately led to exert.

NF-κB family members control transcriptional activity of various promoters of proinflammatory cytokines, cell-surface receptors, transcription factors, and adhesion molecules that are involved in intestinal inflammation[51,52]. Stimuli like oxidative stress, cytokines (interleukin-1, interleukin-6, tumor necrosis factor-α), bacteria and viruses can release NF-κB from its inactive cytoplasmic form to the nucleus[53,54]. Thermal injury has been known to induce hepatic NF-κB expression associated with hepatic cell apoptosis and proliferation[55], but its effect on NF-κB activation in the intestine has never been clarified. Previously, using a thermal injury model in mice, we have shown that CORM-2 plays a pivotal role in inhibition of NF-κB activity in the liver, which subsequently decreases hepatocellular secretion of inflammatory cytokines and burn-related hepatic dysfunction. In this study, NF-κB activity in mid-ileum was elevated by thermal injury, while it was markedly inhibited by administration of CORM-2. These results show that CORM-2 plays, at least partly, an important role in inhibition of NF-κB activity in the small intestine. Therefore, the role of NF-κB activation and the regulation of CORM-2 in thermal-injury-induced intestinal damage requires further study.

In conclusion, the present study serves to clarify the role of CORM-2, one of the novel CORMs, on the mechanisms of anti-inflammation and cytoprotection. Application of CORM-2 to thermally injured mice attenuated PMN accumulation, and prevented activation of NF-κB in the small intestine. This was accompanied by a decrease in expression of ICAM-1, and an increase in expression of HO-1. Taken together, these findings indicate that CORM-released CO modulates gut inflammation in burned mice by interfering with NF-κB activation, and protein expression of ICAM-1 and HO-1, and therefore suppresses the pro-adhesive phenotype of endothelial cells. Further studies are now required to understand the detailed mechanisms of the anti-inflammatory effects mediated by CORMs, and to contribute to the development of a therapeutic approach to protect against gut damage during severe burn injury.

COMMENTS

Background

SIRS and MOF still continue to be leading causes of morbidity and mortality in severe burn patients. The intestine is considered to be the critical organ in the development of organ dysfunction in trauma, burn and intensive care unit patients. Thermal injury is accompanied by complex events that exert deleterious effects on various organs, such as the small intestine, distant from the original burn wound. Following thermal injury, the small intestine is subjected to ischemia, and consequently, especially during burn resuscitation, reperfusion injury occurs. Intestinal ischemia-reperfusion results in organ injury through both tissue hypoxia and reperfusion phenomena mediated by neutrophils. A variety of cytokines are released into the microcirculation by neutrophils, endothelial cells and monocytes during hypoxia and reperfusion. Although the pathophysiological basis of organ damage remains unclear, there is increasing evidence that leukocyte infiltration into intestinal tissue plays an important role in bacterial or endotoxin translocation, and development of SIRS after thermal injury.

Research frontiers

Major burns alter immune function, which produces an imbalance between pro- and anti-inflammatory cytokine synthesis, and increases susceptibility to post-burn infection and sepsis. Also, severe burns cause damage to several organs distant from the original burn wound, which leads to MOF, a serious clinical problem. The intestine is one of the most sensitive tissues to ischemia and reperfusion induced by thermal injury. PMNs may play an important role in ischemic injury, and reperfusion of the intestine is associated with accumulation of PMNs in the intestinal tissue. It has been suggested that tissue accumulation of PMNs is a key event that determines the severity of ischemia-reperfusion injury.

Innovations and breakthroughs

Our study is believed to be the first to observe that CORM-released CO attenuates leukocyte infiltration in the small intestine of thermally injured mice, and the possible mechanisms involved.

Applications

Our research observed that CORM-released CO attenuates leukocyte infiltration in the small intestine of thermally injured mice by interfering with NF-κB activation and protein expression of ICAM-1, and therefore suppresses the pro-adhesive phenotype of endothelial cells. This may have a significant clinical impact in the future.

Terminology

CORMs: transitional metal carbonyls that have been identified as potential CO-releasing molecules with the potential to facilitate the pharmaceutical use of CO by delivering it to tissues and organs.

Peer review

This is a well-written paper that suggests the benefit of CORMs after burn injury. Although the mechanism remains to be determined, I think it may be suitable for publication in WJG. It will be nice to detail other experiments using other doses of CORMs.

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RAPID COMMUNICATION

Heterozygous nucleotide-binding oligomerization domain-2 mutations affect monocyte maturation in Crohn's disease

Marilena Granzotto, Elisa Fabbro, Massimo Maschio, Stefano Martelossi, Sara Quaglia, Alberto Tommasini, Gianni Presani, Alessandro Ventura

Marilena Granzotto, Stefano Martelossi, Alberto Tommasini, Gianni Presani, Alessandro Ventura, Institute for Child Health Burlo Garofolo, Trieste 34137, Italy
Elisa Fabbro, Massimo Maschio, Sara Quaglia, Alberto Tommasini, Alessandro Ventura, Department of Sciences of Reproduction and Development, University of Trieste, Trieste, Italy
Supported by the Italian Ministry of Health, No. 150/03
Correspondence to: Dr. Marilena Granzotto, Laboratory of Immunology, Institute of Child Health IRCCS Burlo Garofolo, Via dell'Istria 65/1, Trieste 34137, Italy. granzotto@burlo.trieste.it
Telephone: +39-40-3785216 Fax: +39-40-3785210
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Abstract

AIM: To investigate the function of monocytes in Crohn's disease (CD) patients and to correlate this with disease-associated nucleotide-binding oligomerization domain-2 (*NOD2*) gene variants.

METHODS: Monocytes from 47 consecutively referred CD patients and 9 healthy blood donors were cultured with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), and stimulated with lipopolysaccharide (LPS) or muramyldipeptide (MDP), the putative ligand of *NOD2*.

RESULTS: We found that monocytes from CD patients differentiated *in vitro* to mature dendritic cells (DCs), as determined by immunophenotype and morphology. *NOD2* genotype was assessed in all subjects, and we observed high CD86 expression on immature and LPS-stimulated DCs in *NOD2* mutated CD patients, as compared with *wtNOD2* CD patients and controls. By contrast, CD86 expression levels of DCs induced to maturity with MDP derived from *NOD2*-mutated subjects were comparable to those of normal subjects. The amount of IL-12p70 in patient-cell cultures was larger than in controls after LPS treatment, but not after treatment with MDP.

CONCLUSION: Our results suggest that DCs obtained from patients with mutations in the *NOD2* gene display an activated phenotype characterized by high CD86 expression, but have a diminished response to MDP when compared to the terminal differentiation phase. We speculate that the altered differentiation of monocytes might lead to an imbalance between inflammation and the killing ability of monocytes, and may be relevant to the pathogenesis of CD.

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract, influenced by both environmental factors and genetic predisposition[1-3]. A significant advance in the understanding of its pathogenesis was achieved by the identification of nucleotide-binding oligomerization domain-2 (*NOD2*) as the first susceptibility gene for CD in Caucasian populations[4,5]. This was the demonstration that a frameshift mutation (1007fs) and two nucleotide polymorphisms (R702W and G908R) in the coding region of *NOD2* predispose people to the disease. Since the identification of *NOD2* mutations associated with CD, much attention has been given to the function of monocytes in which this gene is constitutively expressed[6-8].

The idea that the NOD2 protein is involved in the induction of nuclear factor-kappa B (NF-κB) pro-inflammatory signaling pathways in response to bacterial infections may be relevant for understanding the role of intestinal bacteria in CD[9-12]. Indeed, functional assays on CD-associated isoforms of NOD2 have yielded controversial results concerning the production of inflammatory cytokines and responses to bacteria. NOD2 has been shown to act as both an inducer and a regulator of NF-κB and cytokine production[13,14]; more precisely, muramyldipeptide (MDP)-induced activation of NF-κB lacks mononuclear cells in CD patients homozygous for the 1007fs mutation[15]. Conversely, interleukin (IL)-12 production induced by toll-like receptor 2 (TLR2) is negatively regulated in mice by MDP co-stimulation of *NOD2*; although this effect is absent in *NOD2*-/- mice[14]. Moreover, cells obtained from knock-in mice for the 3020insC *NOD2* mutation show enhanced NF-κB activity,

as well as increased production of IL-1β after MDP stimulation[10]. It is noteworthy that this pro-inflammatory phenotype is also associated with an impaired response to *Listeria monocytogenes* challenge[13]. These data show that CD patients' lymphomonocytes have a defect in the stress-induced production of IL-8, which may be responsible for the impaired response to bacteria[16,17].

Taken together, these data suggest a complex pathogenic model of CD, in which genetic factors favor an imbalance between the inflammatory response and the killing of mucosal bacteria. This is similar to the picture observed in some primary immunodeficiencies. Indeed, a histological lesion typical of CD, chronic granuloma, is common also in some deficiencies of the phagocytic immune system such as chronic granulomatous disease, congenital neutropenia and Wiskott-Aldrich syndrome[18,19]. Based on this, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been beneficially used in patients affected by CD, probably through strengthening their natural immunity[20-22], although it shows no direct anti-inflammatory activity.

We thus hypothesized that, besides the inflammatory response itself, the function of the monocyte-derived immune system may be impaired in CD patients because of mutations in the *NOD2* gene. Therefore, we looked for possible defects in monocyte differentiation in CD patients and the relationship with the *NOD2* genotype.

MATERIALS AND METHODS

Patients

The subjects in this study were 47 patients consecutively referred to our institute for CD, 28 males and 19 females, with a mean age of 16.6 (range 4-33) year, and a mean age at diagnosis of 12.8 year (range 1 mo-18 year). All 47 CD patients were sporadic cases. Thirty had active disease and 17 had clinical, echographic and endoscopic remission at the time of analysis. For *NOD2* genotyping, a control group of 69 blood donors was analyzed. For the functional study of monocytes, the control group was 9 healthy adult blood donors (5 males and 4 females, mean age 24.3 years, range 21-38) who tested negative for CD-associated *NOD2* variants. The study was approved by our local independent ethics committee, and informed consent was obtained from all patients (or their parents) and blood donors.

Genetic analysis

Patients were genotyped for R702W and G908R mutations (identified by PCR amplification and enzymatic digestion) and for the 1007fs mutation (analyzed by amplification and sequencing). DNA was extracted from peripheral blood of patients and controls using a Genomix kit (Talent, Italy). PCR reactions were performed using specific primers for the three mutations (sequences are shown in Table 1), Taq polymerase (Amplitac Gold, Applied Biosystems, Foster City, CA, USA) and a thermal cycler Gene Amp 9700 (PE Applied Biosystems). After denaturing at 95℃ for 10 min, amplification was obtained after 35 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s. For just the 1007fs mutation, 10 amplification cycles at 95℃ for 30 s, 53℃ for 30 s, and 72℃ for 30 s, followed by another 35 cycles at 95℃ for 30 s, 53℃ for 30 s (touch down step: decrease of 0.5℃/cycle), and 72℃ for 30 s. A final step at 72℃ for 7 min was used to stop the reactions. A restriction enzyme digestion assay was performed to detect both R702W and G908R using Msp1 and HhaI, respectively. After digestion, the presence of a wild-type allele resulted in an intact fragment, whereas the variant was characterized by two bands. PCR reaction products underwent electrophoresis on 1.5% agarose gels and were visualized by ethidium bromide staining. Sequencing was carried out for 1007fs detection. Reactions were performed with a Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and on an ABI PRISM 3100 Sequence Detector. Subjects with at least one heterozygous CD-associated variant were categorized as *mtNOD2*, while patients with a homozygous wild type *NOD2* sequence were categorized as *wtNOD2*.

Table 1 Sequence primers used for detection of *NOD2* mutations

Primers	Sequence
Arg702W (forward)	5′-GGCGCCCCTGGAATTC-3′
Arg702W (reverse)	5′-CCTCACCCGGTGCAGC-3′
Gly908Arg (forward)	5′-CCCAGCTCCTCCCTCTTTC-3′
Gly908Arg (reverse)	5′-AAGTCTGTAATGTAAACGCCAC-3′
Leu1007fsinsC (forward)	5′-GAATGTCAGAATCAGAAGGG-3′
Leu1007fsinsC (reverse)	5′-GTCTCACCATTGTATCTTCTTTTC-3′

Cell isolation and dendritic cell generation

To generate *ex vivo* dendritic cells (DCs) from patients and controls, mononuclear cells were isolated by Ficoll separation density-gradient centrifugation, resuspended at a concentration of 2-5 × 10^6 cells/mL in complete RPMI-1640 medium containing 0.1% fetal calf serum (FCS), and allowed to adhere to the well surface of 24-well flat bottom plates (Corning, New York, USA) for 30 min at 37℃ in a 5% CO_2 incubator[23]. After washing twice with PBS to remove non-adherent cells, monolayer cells were cultured for DC differentiation in 0.5 mL RPMI-1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 500 ng/mL GM-CSF (Strathmann Biotec AG, Germany), and 500 ng/mL IL-4 (Strathmann Biotec). After a 3-d culture, 100 μL medium was replaced with a fresh one containing the above-mentioned cytokines. Cell morphology was monitored by light microscopy. Analysis of cell surface marker expression was performed on a suspension of cells harvested on d 8.

Stimulation of DCs

On d 6 of culture, immature DCs were further maturated by adding either 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, Italy) plus 500 ng/mL interferon-γ (INF-γ; Strathmann Biotec) or 500 ng/mL MDP (Sigma-Aldrich) plus 500 ng/mL INF-γ for two more days. After 48 h stimulation, cells were harvested and analyzed by flow cytometry.

Flow cytometry analysis

Cell surface marker expression was evaluated by triple

Table 2 Genotype and allelic frequencies for *NOD2* variants in CD patients and controls

	CD (n = 47)	Healthy controls (n = 69)
Genotype		
Hz	17/47 (36.1%)	4/69 (5.8%)
Double Hz	3/47 (6.4%)	0
mtNOD2	20/47	4/69
wtNOD2	27/47	0/69
Allelic frequencies		
R702W	5.30%	1.45%
G908R	7.45%	0.75%
1007fs	8.50%	0.75%

Table 3 CDAI and drug therapy in *mtNOD2* and *wtNOD2* patients

	mtNOD2 (n = 20)	*wtNOD2* (n = 27)
CDAI	24.5	25.2
Active disease	14	16
Steroids	7	9
Methotrexate	1	1
Azathioprine	4	5
Aminosalicylic acid	6	8
Salazopyrine	4	4
Infliximab	1	1
Thalidomide	4	5

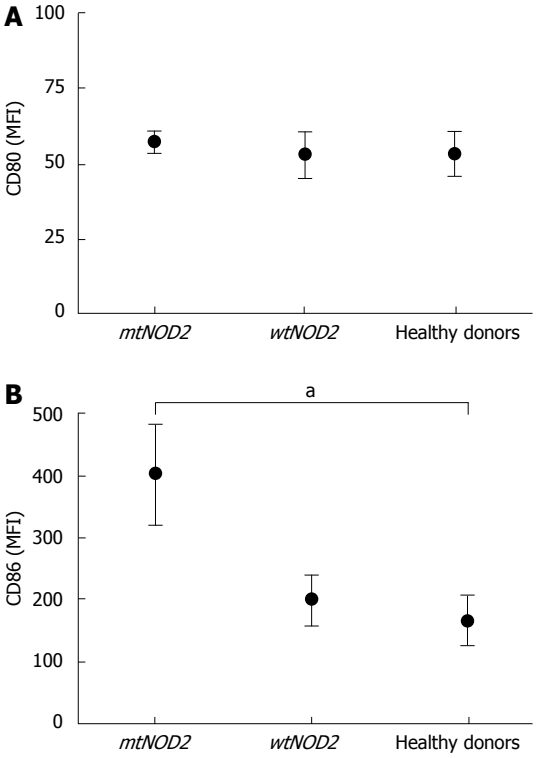

Figure 1 Expression of CD80 (**A**) and CD86 (**B**) in immature DCs, derived from healthy donors, and *mtNOD2* and *wtNOD2* patients. $^{a}P < 0.05$, Student's t test.

immunofluorescence staining with the following monoclonal antibodies: anti-CD80-FITC, anti-CD86-PE, anti-CD83-PE, anti-CD1a-PE, anti-CD33-TC (Caltag Laboratories, Burlingame, CA, USA), anti-CD14-FITC (clone TUK4; Dako Cytomation, Denmark) and anti-HLADR FITC (Becton Dickinson, San Jose, CA, USA). Samples were acquired using a FACScan flow cytometer (Becton Dickinson) and data analysis was performed using CellQuest software (Beckton Dickinson, San Jose, CA, USA). A total of 5000 events were analyzed for each sample.

Cytokine quantification

Supernatants of cell cultures were harvested and stored at -80℃ until measurement of cytokines. Production of IL-12p70 was quantified using an ELISA (Bender MedSystems, Burlingame, CA, USA) according to the manufacturer's instructions.

Statistical analysis

The t test was used to evaluate significant differences. Statistical analysis was performed with the GraphPad Prism program (San Diego, CA, USA), and $P < 0.05$ was considered significant.

RESULTS

NOD2 allelic variants in CD

Seventeen of the 47 CD patients were heterozygous for *NOD2* allelic variants associated with CD, and 3 were double heterozygous for two mutations. The allelic frequencies of R702W, G908R and 1007fs *NOD2* variants in CD patients and healthy donors are shown in Table 2. There was no correlation in our series between disease activity, pharmacological treatment, inflammatory localization and *NOD2* genotype. The mean Crohn's Disease Activity Index (CDAI) and pharmacological treatments are summarized in Table 3.

High CD86 expression in immature DCs derived from NOD2-mutated CD patients

In order to obtain immature DCs, monocytes were first cultured with IL-4 and GM-CSF for 6 d and analyzed for the expression of the two co-stimulatory molecules CD80 and CD86. Under microscopy, the cells from CD patients and controls showed a typical dendritic morphology. Immunocytometry showed no significant differences in CD80 expression in either *mtNOD2* or *wtNOD2* patients as compared with controls. However, CD86 expression was higher in CD patients than in controls, although the difference was not significant; however, it tended to be much higher when *mtNOD2* patients were compared to *wtNOD2* patients and controls ($P = 0.04$) (Figure 1).

Greater effect of LPS as compared to MDP on CD86 upregulation in NOD2-mutated CD patients

After LPS stimulation, terminal differentiation was obtained in DCs in patients and controls. Cells expressed high levels of activation/maturation markers, such as CD83, HLADR and CD1a, without any statistically significant differences among groups (Figure 2). Indeed, DCs from *mtNOD2* patients tended to show higher CD83 expression levels, while those from *wtNOD2* patients presented lower CD1a expression levels. MDP stimulation did not alter these results. Different behavior was shown

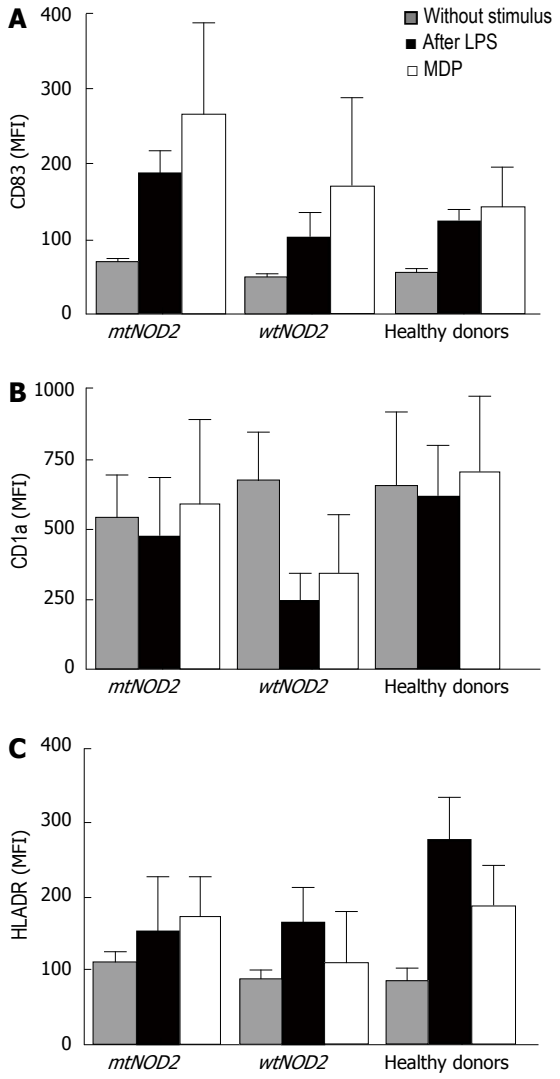

Figure 2 Expression of CD83 (**A**), CD1a (**B**) and HLADR (**C**) in DCs, derived from healthy donors, and *mtNOD2* and *wtNOD2* patients, without stimulus , after LPS or MDP stimulation.

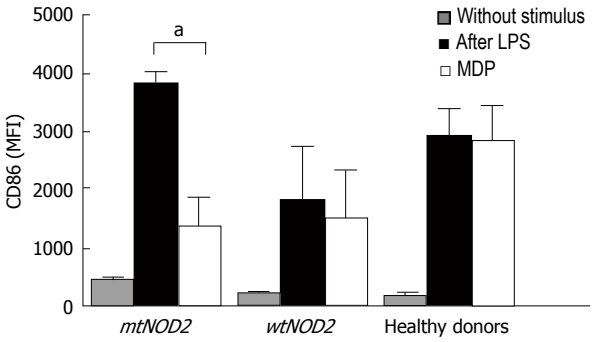

Figure 3 Expression of CD86 in DCs derived from *mtNOD2* and *wtNOD2* patients and healthy donors without stimulus, after LPS or MDP stimulation. [a]$P < 0.05$ LPS- *vs* MDP-stimulated *mtNOD2* patients, Student's *t* test.

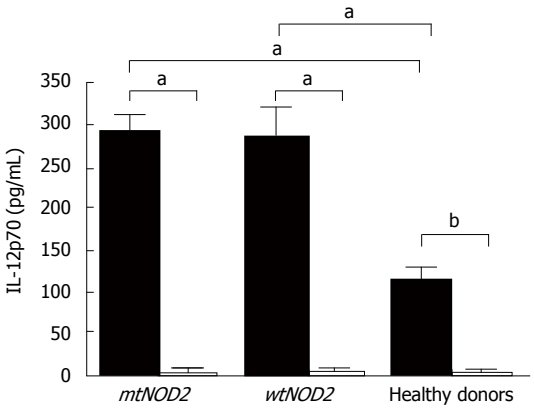

Figure 4 IL-12p70 levels (pg/mL) in monocyte-culture supernatants after stimulation with LPS (■) or MDP (□) in *mtNOD2* and *wtNOD2* patients and healthy controls. [a]$P < 0.05$ LPS- *vs* MDP-stimulated *mtNOD2* patients, LPS- *vs* MDP-stimulated *wtNOD2* patients, LPS-stimulated *mtNOD2* patients *vs* LPS-stimulated healthy donors, LPS-stimulated *wtNOD2* patients *vs* LPS-stimulated healthy donors; [b]$P < 0.01$ LPS- *vs* MDP-stimulated healthy donors; Student's *t* test.

by CD86 in *mtNOD2* patients compared to *wtNOD2* patients and healthy donors (Figure 3). A significantly greater up-regulation of CD86 was shown in *mtNOD2* after LPS stimulation compared to after MDP stimulation ($P = 0.016$). This difference was completely absent in *wtNOD2* and controls.

High levels of IL-12p70 expression in LPS-matured DCs from CD patients

MDP and LPS stimulation of monocyte cultures from patients and healthy donors was evaluated by measuring the production of the bioactive form of IL-12p70 (Figure 4), a major regulatory cytokine of the adaptive immune response. LPS induced higher levels of cytokines in CD patients (whether *mtNOD2* or *wtNOD2*) (mean values ± SD: 291 ± 42 pg/mL in *mtNOD2* and 285 ± 61 pg/mL in *wtNOD2*) in comparison to controls (112 ± 38.2 pg/mL), which showed a statistically significant difference ($P < 0.05$). MDP, on the other hand, was inactive, thus preventing cytokine production in DCs from CD patients (mean values ± SD: 3.2 ± 7.15 pg/mL in *mtNOD2* and 2.8 ± 6.2 pg/mL in *wtNOD2*) and healthy donors (1.94 ± 3.46 pg/mL, $P = 0.0079$).

DISCUSSION

Although several genes involved in CD have been described to date, the pathogenesis of the disease remains largely unknown[24,25]. It is thought that the disease arises from abnormal crosstalk between a changing intestinal flora and the host in the presence of a genetic background able to influence the integrity of the intestinal barrier and/or the functioning of the innate immune response[1,26]. Since the identification of *NOD2* mutations is associated with CD, much attention has been placed on the functions of monocytes in which this gene is constitutively expressed[6]. It has been hypothesized that some slight innate immunity defects may underlie the pathogenesis of CD. Kramer *et al* have recently identified a defect in response to MDP stimulation of DCs obtained from patients with homozygous 3020insC *NOD2* mutations, which suggests that a defect in the production of cytokines like IL-10 plays a role in the pathogenesis of the disease, by diminishing immune tolerance to intestinal bacteria.

In this work, we tested the ability of monocytes from CD patients to differentiate *in vitro* into DCs. The results indicated that monocytes differentiated into DCs using conventional stimuli. However, DCs obtained from CD patients with *mtNOD2* showed some differences in their expression of activation antigens before and after stimulation. These differences are not likely to depend on disease activity or drugs, as the *NOD2* genotype did not influence such aspects in our study (as in other published series). The most striking difference is the elevated expression of CD86 on immature *mtNOD2* DCs. Moreover, the *mtNOD2* group displayed a greater difference in CD86 up-regulation after LPS as compared to MDP stimulation. This was partially in agreement with the observations of Kramer *et al*[27] in patients with a homozygous 3020insC *NOD2* mutation, whose DCs failed to up-regulate CD80 and CD86 upon MDP stimulation. In our experiment, it was of particular interest that the expression of these activation markers was higher in immature cells, thus suggesting a continuous stimulation of these cells *in vivo*. Indeed, a high expression of activation markers on peripheral blood monocytes from CD patients has been reported[28]. Moreover, it is noteworthy that we could see these differences in heterozygous *mtNOD2* subjects when compared to *wtNOD2* CD patients and controls. This suggests some interference of mutated and normal proteins, perhaps in the process of homodimerization[29].

In conclusion, we showed that DCs obtained from patients with mutations in *NOD2* tended to be more activated than those obtained from *wtNOD2* and controls. However, during terminal differentiation, these DCs were less responsive to MDP compared to LPS. This may be the cause of an imbalance between inflammation and the killing ability of monocytes that may be relevant to the pathogenesis of CD.

COMMENTS

Background

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. The incidence of the disease is rising in countries with improving socio-economic conditions. Although several hypotheses have been raised about the environmental factors involved in the risk of CD, the issue remains unresolved. Genetic data have recently identified genes responsible for susceptibility to CD and provided new tools for studying interactions between the immune system and the environment in the pathogenesis of CD.

Research frontiers

Several lines of evidence suggest that the disease may arise from an altered sensing of the microbial environment in the gut by the innate immune system. (1) CD patients produce antibodies against common intestinal commensal microbes. (2) Elemental diet is an effective treatment for CD. (3) Variants of the *NOD2* gene, which is involved in control of inflammatory responses to bacteria in monocytes, confer susceptibility to CD. (4) An inflammatory disease of the gut is typical of most primary immunodeficiencies involving the innate system. (5) Granulocyte monocyte-colony stimulating factor (GM-CSF) has been shown to ameliorate CD. However, the study of *NOD2* mutants has brought controversial results regarding the interpretation of CD pathogenesis. The study of the behavior of CD monocytes can help clarify this issue.

Innovations and breakthroughs

Most previous studies have analyzed *NOD2* mutations in cellular models, and have concluded that the consequences of *NOD2* mutation are either pro-inflammatory (activation of nuclear factor-κB) or a deficiency in response to bacteria. However, the situation *in vitro* is more complex. We demonstrated abnormal behavior of monocytes from CD, with an easier capacity to become activated but with only minor ability to complete differentiation. While other studies have shown a defective differentiation only for *NOD2* homozygous patients, we demonstrated that some differences may be present also in heterozygous patients. We hypothesized that the altered differentiation of monocytes might lead to an imbalance between inflammation and the killing ability of monocytes, and therefore is probably relevant to the pathogenesis of CD.

Applications

This study can help in the understanding of the therapeutic paradox of a disease that can be treated both with anti-inflammatory drugs and with cytokines able to strengthen the innate immune response. Further studies will be needed to determine those CD patients who are more likely to receive benefits from these two different treatment options.

Terminology

wtNOD2 represents the form of the gene without mutations. DCs are the most effective cells in presenting antigens to and stimulating T cells. They can develop from monocytes and histiocytes. Muramyldipeptide (MDP) is the minimal bioactive peptidoglycan motif common to all bacteria, and it is the essential structure required for adjuvant activity in vaccines.

Peer review

Generally this is a well written paper and provides further evidence concerning the effects of *NOD2* mutants on immune responses to bacterial stimuli.

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RAPID COMMUNICATION

Accurate positioning of the 24-hour pH monitoring catheter: Agreement between manometry and pH step-up method in two patient positions

Mehmet Fatih Can, Gokhan Yagci, Sadettin Cetiner, Mustafa Gulsen, Taner Yigit, Erkan Ozturk, Semih Gorgulu, Turgut Tufan

Mehmet Fatih Can, Gokhan Yagci, Sadettin Cetiner, Taner Yigit, Erkan Ozturk, Turgut Tufan, Departments of General Surgery, Gulhane Military Medical Academy, Etlik, Ankara, Turkey
Mustafa Gulsen, Semih Gorgulu, Gastoenterology and Hepatology, Gulhane Military Medical Academy, Etlik, Ankara, Turkey
Mehmet Fatih Can, Gokhan Yagci, Sadettin Cetiner, Mustafa Gulsen, Taner Yigit, Erkan Ozturk, Semih Gorgulu, "Reflux Research Group", Gulhane Military Medical Academy, Etlik, Ankara, Turkey
Correspondence to: Mehmet Fatih Can, MD, Departments of General Surgery, Gulhane Military Medical Academy, Kent Koop Mh. 11.Cd. Guneyce Merkez Sit. A/10, 06370, Batikent Ankara, Turkey. mfcan@gata.edu.tr
Telephone: +90-505-4568779 Fax: +90-312-3045100
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Abstract

AIM: To investigate the agreement between esophageal manometry and pH step-up method in two different patient positions.

METHODS: Eighteen subjects were included in the study. First, the distance from the nose to the proximal border of the lower esophageal sphincter (LES) was measured manometrically. Then a different investigator, who was blinded to the results of the first study, measured the same distance using the pH step-up method, with the patient in both upright and supine positions. An assessment of agreement between the two techniques was performed.

RESULTS: In the supine position, the measurement of only one subject was outside the range accepted for correct positioning (≤ 3 cm distal or proximal to the LES). In the upright position, errors in measurement were recognized in five subjects. Bland-Altman plots revealed good agreement between measurements obtained manometrically and by the pH-step up method with the patient in the supine position.

CONCLUSION: In the case of nonavailability of manometric detection device, the pH step-up method can facilitate the positioning of the 24 h pH monitoring catheter with the patient in the supine position. This should increase the use of pH-metry in clinical practice for subjects with suspected gastroesophageal reflux disease if our results are supported by further studies.

Key words: pH monitoring; Esophageal manometry; pH step-up method; Gastroesophageal reflux

Can MF, Yagci G, Cetiner S, Gulsen M, Yigit T, Ozturk E, Gorgulu S, Tufan T. Accurate positioning of the 24-hour pH monitoring catheter: Agreement between manometry and pH step-up method in two patient positions. *World J Gastroenterol* 2007; 13(46): 6197-6202

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INTRODUCTION

Although the number of patients with a confirmed diagnosis with gastroesophageal reflux disease (GERD) is low, the condition is thought to affect many more people worldwide[1]. The evaluation of patients with reflux symptoms often requires using an advisable diagnostic tool both for distinguishing between physiologic and pathologic reflux, and for the purpose of treatment. New diagnostic tests (multichannel impedance monitoring, wireless pH monitoring capsules, *etc.*) have been developed and have become popular over the last decade, especially in leading industrial countries[2]. However, the conventional 24 h ambulatory pH monitoring continues to be the most common diagnostic test for determining pathologic acid reflux in developing countries. Traditionally, the distal pH sensor of the monitoring catheter is positioned 5 cm above the proximal border of the lower esophageal sphincter (LES), since the threshold used to discriminate the diagnostic cut-off level of acid exposure has been validated at this point[3]. The most popular method used for detecting this location is prior esophageal manometry. However, several measures have been employed to accurately determine the location of the LES, since manometric measurement (despite being essential to recognize motility disorders) is time consuming, is an invasive procedure[4-10], is uncomfortable for patients, and

leads to an increase in the cost of the diagnostic work-up.

In the past, the pH step-up method has been used for this purpose, but the results obtained were conflicting[5,11]. It is well known that gravity and body position have an effect on organ position, and in particular abdominal contents[12]. In the present study, we investigated the influence of body position on the location of the pH monitoring catheter during the pH step-up process. We hypothesized that localizing the pH probe with the patient in a supine position was more accurate since it is the same position as is used in manometric measurement.

MATERIALS AND METHODS

Preparation for the study

The study was conducted between May 2004 and July 2005, in a Military Medical Academic Hospital, in the GI Endoscopy and Manometry Laboratory of the General Surgery Department. Eighteen patients with reflux symptoms, but without hiatus hernia, (ten male and eight female), ranging in age from 22 to 68 (median age 46) years, and referred for esophageal manometry and esophageal pH monitoring constituted the study group. All esophageal tests were performed by two physicians, each of whom was blind to the results obtained by the other, however, all therapeutic decisions were made by a multidisciplinary gastroesophageal reflux team.

The tests were explained in detail to each patient, and written consent was obtained. All patients had been investigated endoscopically prior to the study, and only two had esophagitis. Patients with esophagitis had grade B disease according to the Los Angeles Classification[13] and there was no evidence of any serious disease (Barrett's esophagus, carcinoma *etc*.). Esophageal pH monitoring determined the presence of GERD in eleven cases (GERD group) while physiologic acid reflux was observed in the remaining subjects (Non-GERD group). All medications which could potentially impact esophageal motility, LES pressure and acid reflux were discontinued fifteen days prior to the tests.

Study protocol

An eight-lumen polyvinyl catheter with an external diameter of 4.5 mm [four lumens arranged 90º from each other for the most distal openings on the same circle, and the remaining four lumens with openings 5, 10, 15 and 20 cm proximal to the distal one respectively (Solar, MMS B.V. Enschede, The Netherlands)] was used for esophageal manometry. Once perfused and filled with distilled water, each lumen was connected to a pressure transducer-recorder system. Perfusion was maintained by fluid flow of 1 mL/min. After a six hour fast, patients were admitted to the laboratory. Topical anesthesia was applied, and with the patient in the supine position the catheter was introduced through the nose and into the stomach. The distance from the proximal margin of the LES to the nostril, the mean resting pressure and length of the LES, receptive relaxation of the LES, waves of esophageal peristalsis stimulated by wet swallow, and upper esophageal sphincter location were detected by using the stationary pull-through method. Each of the pulling steps was at intervals of 1 cm. The actual location of the proximal margin of the LES was based on the mean location of four radially oriented openings. The catheter was removed after measurements of the upper esophageal sphincter resting pressure and the location site was recorded.

After obtaining the manometric values, a different investigator who was blind to these findings performed the pH study. For this purpose, we used a double electrode ambulatory pH monitoring catheter [first sensor on the tip, and another 15 cm above the first (pH probe meter, MMS B.V. Enschede, The Netherlands)], calibrated with buffer fluids at pH 7.0 and 1.0, immediately prior to the test. The catheter was introduced into the stomach through the nose, with the patient in the upright position and breathing shallow. After confirmation of acidic pH, the catheter was withdrawn gradually until an abrupt rise in pH to > 4.0 (pH step-up) was detected. The pH readings were used to indicate the proximal margin of the LES at the esophagogastric junction. To confirm this point, the catheter was withdrawn at least a further 10 cm and then re-inserted into the stomach. The process was repeated two more times. The mean value of the three readings was used when there was a difference in the three measurements.

All tests after esophageal manometry were repeated with patients in the supine position. Following this stage, the first investigator rejoined the study and positioned the distal electrode of the catheter 5 cm above the upper margin of the LES, based on the actual LES location determined by manometric study. A chest x-ray was obtained to ensure against any bends or rolls. If any bend, roll, or dislocation was noted, the catheter location was re-confirmed after making the necessary corrections. Finally, ambulatory 24 h pH monitoring was initiated, with comments made on the usage rules of the recording machine.

Statistical analysis

The results of statistical analysis are noted in the text as mean ± standard deviation (SD) except for age. To analyze the significance of the results, the SPSS version 11.0 software package program (SPSS Inc, Chicago, IL, USA) was used. All data obtained from manometric measurements were compared between patients with pathologic and physiologic reflux using the Mann-Whitney-*U* test. The results of measurement and analysis with respect to esophageal motility above the LES and upper esophageal sphincter functions were not included in this report. An agreement between methods was assessed using the Bland-Altman analysis. This method tests agreement between two measurements, one of which is generally accepted as the gold standard. In addition, correlation between distances measured manometrically and by pH step-up method was calculated using Spearman's rho test. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Patients referred for esophageal manometry and 24 h ambulatory pH monitoring were identified. None of

Table 1 Comparative results of GERD and non-GERD groups

Variables (mean ± SD)	**GERD group**	**Non-GERD group**
n (male/female)	11 (6/5)	7 (4/3)
Age (yr)	48 (26-68)	43 (22-61)
LES length (cm)	3.27 ± 0.9	2.86 ± 0.7
LES resting pressure, mmHg	10.82 ± 2.6	18.43 ± 4.0[a]
Distance: nose to the PB-LES, cm (by manometry)	42 ± 3.8	41.7 ± 3.5
Distance: nose to the PB-LES, cm (by pH step-up in upright position)	44.7 ± 2.6	43.8 ± 3.5
Distance: nose to the PB-LES, cm (by pH step-up in supine position)	43.4 ± 3.0	42.1 ± 3.3
Clear detection of receptive relaxation of the LES (*n*)	9/11	7/7

[a]$P < 0.05$ *vs* GERD group (Mann-Whitney-*U* test). LES: Lower esophageal sphincter; PB-LES: Proximal border of the LES.

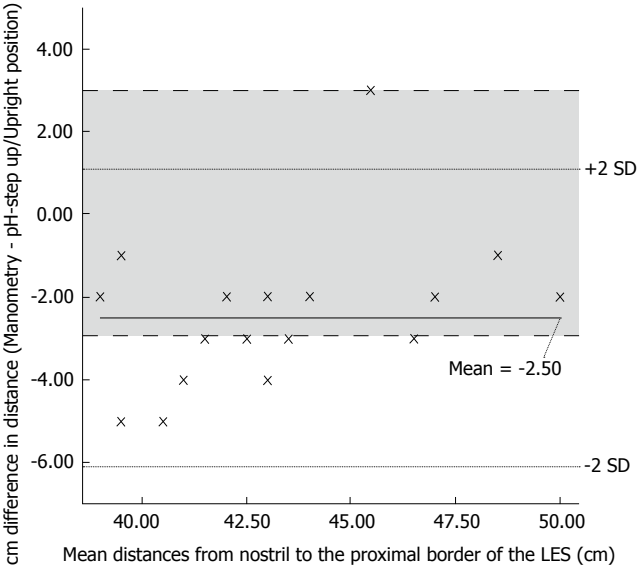

Figure 1 Bland-Altman scatter graph plotted to assess agreement between measurements obtained manometrically and by the pH step-up method with patients in the upright position. Grey area surrounded by longer dashed line indicates clinically acceptable limits.

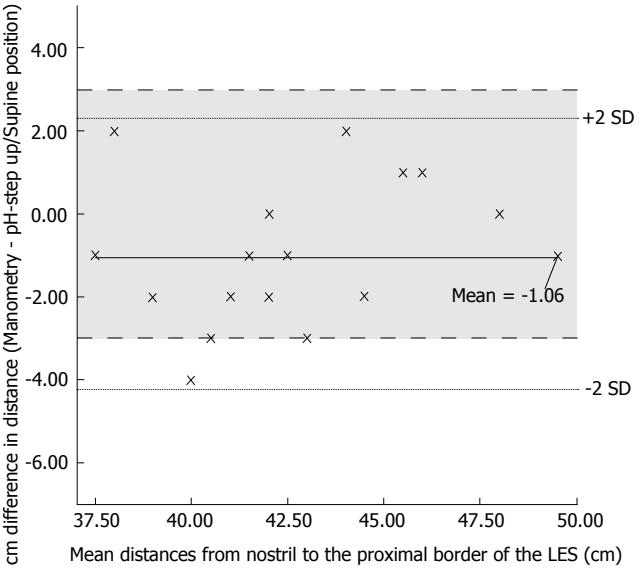

Figure 2 Bland-Altman scatter graph plotted to assess agreement between measurements obtained manometrically and by the pH step-up method with patients in the supine position. Grey area surrounded by longer dashed line indicates clinically acceptable limits.

the subjects had a hiatus hernia, an esophageal motility disorder, or dysfunction of the upper esophageal sphincter. According to the DeMeester reflux scoring system[14], eleven patients had acid reflux above the pathologic level; while the remaining subjects experienced reflux within the physiologic range (data not provided).

The results of comparison between the LES length and the mean resting pressure of the LES are given in Table 1. The LES length was 3.27 ± 0.9 in the GERD group and 2.86 ± 0.7 in the non-GERD group ($P = 0.268$). There was a significant difference between the mean resting LES pressures (10.82 ± 2.6 for GERD group and 18.43 ± 4.0 for non-GERD group, $P = 0.02$). In addition, a receptive relaxation at the level of LES was clearly observed in all but two subjects whose mean resting LES pressures were comparatively lower (7 and 8 mmHg, respectively).

In both study groups, the distance from the nose to the proximal border of the LES, detected manometrically was in the range of 37 to 49 (average 41.89 ± 3.58) cm. The same distance measured by the pH step-up method ranged between 40 and 51 (average 44.39 ± 2.95) cm in the upright position, and between 37 and 50 (average 42.94 ± 3.09) cm in the supine position. Bland-Altman (bias) statistics showed a good agreement between the distances measured manometrically and by the pH step-up method. This agreement was found within clinically acceptable limits (difference ± 3 cm) in measurements obtained with patients in the supine position [difference (mean ± SD): -1.06 ± 1.76, CI 95% for supine position and -2.5 ± 1.82, CI 95% for upright position] (Figures 1 and 2). When the differences are analyzed based on the individual results, a difference of > 3 cm between measurements obtained manometrically and by the pH step-up method in a supine position was observed in only one subject, where as there were five subjects who had a difference > 3 cm in the measurements performed in the upright position. A strong correlation was also noted between the two methods (correlation coefficient: 0.842, $P < 0.0001$ for manometry/pH step-up in the upright position, and correlation coefficient: 0.891, $P < 0.0001$ for manometry/pH step-up in the supine position, respectively, using the Spearman's rho test). A comparison of the GERD *vs* non-GERD patients with respect to the measurement obtained with each method-manometry, pH step-up in an upright position, and pH step-up in a supine position-showed no statistically significant difference between the two groups.

DISCUSSION

Our comparative study of LES function in patients with pathologic *vs* physiologic reflux are in agreement with established data regarding the pathogenesis of GERD. We observed a significant difference in the LES resting pressures of both groups, but not in the LES lengths.

Similar results were reported in a previous study[15]. Another report demonstrated that patients with reflux esophagitis had a lower minimum LES pressure compared with healthy subjects[16]. However, we could not determine the receptive relaxation of the LES during wet swallow in two patients with GERD. This was perhaps due to the presence of very low resting pressures, which made measurement of the relaxation pressure difficult. The results of pH monitoring and the management of patients with pathologic reflux have not been provided in this report.

For an accurate positioning of a pH electrode, esophageal manometry is widely accepted as the gold standard. Almost all of the other methods recommended for precise pH electrode placement (endoscopy, fluoroscopy, transnasal fiberoptic laryngoscopy, *etc.*), require interventions which increase the cost and are more invasive. It is for this reason that we recommend the pH step-up method, which aims at identifying the proximal border of the LES and only requires the use of pH monitoring catheter. Our results are based on the detection of an abrupt rise in the pH as the catheter is withdrawn from the acidic pH of the stomach into the neutral (pH > 4) environment of the esophagus. In contrast to other studies[5,17,18], some reports have stressed that the pH step-up method cannot be used for positioning of the pH electrode in the esophagus[11,19,20]. One of these studies by Marples and colleagues[20], suggested that false positioning of the pH electrode may occur (they detected incorrect positioning of 10 cm above and 16 cm below the LES) if the pH step-up method is used. Pehl and co-workers interpreted this inter-individual variance as a methodological bias, and have explained this on the basis of a lower position of the electrode, presumably because of the inability to detect acidic content secondary to the location of the electrode in the fundic air, when the patient is in the upright position[18]. This explanation supports our results, as it suggests that in the supine position, the withdrawal process may help in the correct positioning of the pH electrode. In fact, it should be recognized that a significant number of physicians interested in GERD and related illnesses have to manage such patients without esophageal manometry, since this test is still not commonly available, especially in community hospitals and small medical centers in developing countries[21]. For example, in the city of Ankara (the second largest city in Turkey), there are ten medical departments using esophageal pH monitoring in adult patients. However, adult participants are investigated by manometry in only five of these centers. This rate is similar to that in pediatric centers; therefore, a correct guide to the use of the pH step-up method is required.

We proposed to answer the following three questions with this study: (1) Could manometric determination of the LES level be safely replaced by the pH step-up method? (2) Does the degree of reliability change with the position of patient during pull-through?, and (3) Is there any difference in the measurements of GERD *vs* non-GERD patients? Esophageal manometry is generally performed when the patient is in a supine position and motionless; this provides the most accurate results from level-oriented pressure sensors. These sensors are properly calibrated prior to each measurement. When an individual reclines from the sitting to the supine position, the fundic air is dispersed throughout the stomach[22], and thus most of the acidic fluid content of the stomach comes into contact with inferior surface of the LES. Therefore, we hypothesized that the distal electrode of the pH monitoring catheter more precisely registers the pH change during the withdrawal process when the patient is in the supine position. Furthermore, as previously reported, LES pressure is higher in the supine position compared to the sitting position, particularly in patients with reflux esophagitis[22]. This may facilitate determining the pH change by preventing the escape of acid fluid into the esophagus, especially in patients whose LES pressure is very low. Despite this, reflux can occur when the patient is in a supine position. Therefore, to avoid a flawed measurement during a possible reflux episode, we repeated the withdrawal process two more times. In another report supporting the reliability of our measurements, the supine position was shown to have no greater influence on the amount of possible acid exposure than that of a 20 degree head-up position in healthy individuals, even if the stomach was full[23]. A previous study by Decktor *et al* showed that the placement of the esophageal pH monitoring catheter across the gastroesophageal junction did not increase gastroesophageal reflux[24]. These findings support our hypothesis with respect to the trustworthiness of the method.

Another possible reason for the difference in the measurements between the upright and supine positions is the effect of gravity on organ placement. It is well known that gravity, plays a significant role in posture and the proprioceptive location of body parts[12]. This effect may change an organ's location if it is sufficiently free from adjacent structures. There is a continuous movement of internal organs, caused by factors such as breathing, postural changes, and muscle contractions, which affects the abdominal contents as well as the diaphragm[25,26]. Although less than that compared to intra abdominal organs, there is measurable movement with changes in posture of organs within the ribcage. The pericardium, a structure that is adherent to adjacent tissues, has been shown to be mobile in the sagittal plane[27]. In light of these observations, it is possible that the LES may have a similar posture-related movement. This would help explain the difference in the measurement between the nostril and the proximal border of LES in the two positions. The present study showed no difference in the distance between nose and the proximal border of the LES between patients with and without GERD. These results were obtained both by the pH step-up technique as well as by the manometric method (Table 1). This finding is of critical importance as it indicates that the pH step-up method can be used in both GERD and non-GERD patients.

Measuring the exact location of the proximal border of the LES is presumably impossible because the LES is a ring that is localized in a diagonal plane in association with complex vector volumes[28]. Therefore, a difference in distance of up to 2 cm may be determined by different radially-oriented openings during manometric measurement. For this reason, an electrode positioned

by any method, at 3 cm above or below the manometric position, is commonly accepted as accurate[11,18]. However, if the electrode is positioned with a difference of 5 cm or more, a significant error in acid detection can occur. Anggiansah and co-workers reported that in nine out of twenty GERD patients, the clinical diagnoses according to the DeMeester reflux scoring system, was altered if the pH electrode was placed 10 cm above the LES[29]. Another study showed that there was a two-fold greater measurement of reflux events if an electrode was positioned at 1 cm compared with 5 cm above the gastroesophageal junction[30]. According to our results, all but one pH step-up measurement (successful in 17 of 18) made in the supine position was in the acceptable range (± 3 cm), whereas five measurements failed to determine the acceptable pH-step up location (successful in 13 of 18) in the upright position. Bland-Altman agreement plots demonstrated the superiority of measurements obtained in the supine position.

These findings clearly indicate the need for accurate placement of the ambulatory pH monitoring catheter with the patient in a supine position rather than in an upright position. However, the relatively small number of patients that were investigated may restrict the power of this conclusion. The total number of individuals tested was low because the study was designed to include subjects without hiatus hernia. Therefore, additional prospective, double-blind trials with larger number of subjects are needed for better understanding of this issue. Although patient position during diagnostic work-up may seem to be of little significance, the overall number of individuals who suffer from suspected reflux symptoms makes this issue quite important. In our opinion, physicians who manage patients with GERD should use this technique in clinical practice, especially in centers where manometric devices are not available.

In summary, we consider 24-h pH monitoring as the most valuable diagnostic tool in GERD; until such time as new methods now under development become widely available. Esophageal manometry is still the most reliable method for determining the proximal border of the LES. However, it has several limitations to its routine use. Hence, we recommend an easy method of placement of the ambulatory pH monitoring catheter. To our knowledge, this is the first study designed to assess the effect of patient's position on pH monitoring catheter location by an evidence-based clinical trial. Further studies with larger number of subjects are needed to confirm these results. We conclude that if the catheter is positioned when the patient is in a supine position rather than an upright position, the results obtained are more accurate. This may increase both the use of pH measurements without manometry and improve the diagnosis rate of GERD in developing regions of the world.

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COMMENTS

Background

24-h pH monitoring remains the most crucial test to determine pathologic acid reflux in gastroesophageal reflux disease. Accurate placement of 24-h pH monitoring catheter requires prior esophageal manometry that enables physicians to precisely detect the upper border of the lower esophageal sphincter (LES). Manometric measurement is a relatively invasive procedure, is uncomfortable to patients, and leads to increase in the cost as well as the time spent on investigating each patient. The pH step-up method has been recommended for this purpose; however, published reports provide conflicting results on its usefulness. In the present study, we investigated the influence of patient posture on the pH step-up method, which resulted in improvement in the accuracy of the test.

Research frontiers

In an attempt to find an alternative method to esophageal manometry, different workers have recommended other techniques such as endoscopy, fluoroscopy and transnasal fiberoptic laryngoscopy; however, all of these add new difficulties and additional cost to the diagnostic workup. By contrast, the pH step-up method only requires the use of a monitoring catheter, however any alteration in the location of the pH sensor results in false measurement. This fundamental aspect of the study led us to the finding that the location and level of the pH sensor in the esophagus can alter with posture. To date, this finding has not been reported; and therefore, we decided to prospectively investigate the effect of patient posture on the position of pH monitoring catheter.

Innovations and breakthroughs

The present study revealed that the pH monitoring catheter could be more accurately positioned when the examination is performed with patient in a supine position. Bland-Altman (bias) statistics showed a good agreement between the distances measured manometrically and by the pH step-up method; however, this agreement was found within clinically acceptable limits (difference ± 3 cm) only with the measurements obtained with patients in the supine position. The possible factors and effects leading to this result are discussed in the present article.

Applications

The main debate about the use of the pH step-up method is focused on the ability to accurately position the monitoring catheter. The results of the present study suggest that examination carried out with the patient in the supine position increases the likelihood of accurate placement. Despite these encouraging findings, because of the small number of patients included in the present study, further trials with larger study population will be required to confirm our findings. If confirmed, this technical step will help make 24-hour pH monitoring a routine procedure. We believe that catheter placement for ambulatory pH monitoring can be more easily managed by using the pH step-up method.

Terminology

pH step-up: defines a sudden increase in the pH as the pH monitoring catheter is withdrawn from the acidic content of the stomach into the neutral (pH > 4) environment of the esophagus. It indicates that the pH sensor has crossed the gastroesophageal junction. Blant-Altman analysis: This test is used to compare the bias (the mean of the differences) and limits of agreement (bias ± 2 SD of bias) between two methods, one of which is accepted as the gold standard.

Peer review

In this manuscript, the authors ascertained the accuracy of the step-up method in intra-esophageal pH monitoring. The study was well performed and the conclusion is clear and very interesting.

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RAPID COMMUNICATION

Combination of allopurinol and hyperbaric oxygen therapy: A new treatment in experimental acute necrotizing pancreatitis?

Bilgin Comert, Ahmet Turan Isik, Sezai Aydin, Ergun Bozoglu, Bulent Unal, Salih Deveci, Nuket Mas, Esref Cinar, Mehmet Refik Mas

Bilgin Comert, Division of Intensive Care, Departments of Internal Medicine, Gulhane School of Medicine, Ankara 06018, Turkey
Ahmet Turan Isik, Ergun Bozoglu, Division of Geriatric Medicine, Department of Internal Medicine, Gulhane School of Medicine, Ankara 06018, Turkey
Sezai Aydin, Department of Surgery, Guven Hospital, A. Ayranci, Ankara 06018, Turkey
Bulent Unal, Department of Surgery, Numune Training Hospital, Sihhiye, Ankara 06018, Turkey
Salih Deveci, Department of Pathology, Gulhane School of Medicine, Ankara 06018, Turkey
Nuket Mas, Department of Anatomy, Medical Faculty, Baskent University, Ankara 06018, Turkey
Esref Cinar, Department of Infection Disease, Gulhane School of Medicine, Ankara 06018, Turkey
Mehmet Refik Mas, Department of Internal Medicine, Gulhane School of Medicine, Ankara 06018, Turkey
Correspondence to: Dr. Ahmet Turan Isik, Associate Professor, Division of Geriatric Medicine, Departments of Internal Medicine, Gulhane School of Medicine, GATA Geriatri BD, Etlik, Ankara 06018, Turkey. ahmetturanisik@yahoo.com
Telephone: +90-312-3043121 Fax: +90-312-3044000
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Abstract

AIM: To investigate the individual and combined effects of allopurinol and hyperbaric oxygen (HBO) therapy on biochemical and histopathological changes, oxidative stress, and bacterial translocation (BT) in the experimental rat acute pancreatitis (AP).

METHODS: Eighty-five Sprague-Dawley rats were included in the study. Fifteen of the eighty-five rats were used as controls (sham, Group Ⅰ). AP was induced via intraductal taurocholate infusion in the remaining seventy rats. Rats that survived to induction of acute necrotizing pancreatitis were randomized into four groups. Group Ⅱ received saline, Group Ⅲ allopurinol, Group Ⅳ allopurinol plus HBO and Group Ⅴ HBO alone. Serum amylase levels, oxidative stress parameters, BT and histopathologic scores were determined.

RESULTS: Serum amylase levels were lower in Groups Ⅲ, Ⅳ and Ⅴ compared to Group Ⅱ (974 ± 110, 384 ± 40, 851 ± 56, and 1664 ± 234 U/L, respectively, $P < 0.05$, for all). Combining the two treatment options revealed significantly lower median [25-75 percentiles] histopathological scores when compared to individual administrations (13 [12.5-15] in allopurinol group, 9.5 [7-11.75] in HBO group, and 6 [4.5-7.5] in combined group, $P < 0.01$). Oxidative stress markers were significantly better in all treatment groups compared to the controls. Bacterial translocation into the pancreas and mesenteric lymph nodes was lower in Groups Ⅲ, Ⅳ and Ⅴ compared to Group Ⅱ (54%, 23%, 50% *vs* 100% for translocation to pancreas, and 62%, 46%, 58% *vs* 100% for translocation to mesenteric lymph nodes, respectively, $P < 0.05$ for all).

CONCLUSION: The present study confirms the benefit of HBO and allopurinol treatment when administered separately in experimental rat AP. Combination of these treatment options appears to prevent progression of pancreatic injury parameters more effectively.

Key words: Experimental pancreatitis; Allopurinol; Hyperbaric oxygen

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INTRODUCTION

Acute pancreatitis (AP) is an untreatable condition with a wide clinical spectrum ranging from a mild, self-limited disease to severe organ failure[1]. Translocation of enteric bacteria is the most important cause of infection in the pancreatic tissue, and subsequent events such as sepsis and related complications[2,3]. Currently, several treatment options have been proposed for the septic complications of AP.

Hyperbaric oxygen (HBO) therapy has been investigated in several experimental and clinical conditions which cannot be treated with currently available medical

or surgical options[4,5]. HBO has been shown to have bactericidal activity against anaerobic bacteria[6]. In addition, HBO reduces the incidence of bacterial translocation[7]. It also lowers nitric oxide production and enhances several activities including bactericidal action of neutrophils, angiogenesis and wound healing[8]. Lin *et al* showed that repeated HBO therapy in endotoxic rats reduced inflammatory mediators and free radicals, as well as mortality[9]. In a rat model of tourniquet-induced ischemia-reperfusion skeletal muscle injury, HBO attenuated the reperfusion-induced increase in catalase activity and malondialdehyde (MDA)[10]. These studies demonstrated that HBO treatment, especially when administered in repeated doses has antioxidant rather than oxidative effect.

Xanthine oxidase plays an important role in the migration of microorganisms from the intestinal lumen to intra-abdominal spaces in pathological conditions, an event termed bacterial translocation (BT)[11,12]. Allopurinol (ALPL) has antioxidant properties, and previous studies have shown that antioxidant therapy reduces tissue injury and bacterial translocation in experimental pancreatitis[13,14].

The present study was carried out to investigate the individual and combined effects of ALPL and HBO on biochemical and histopathological changes, oxidative stress, and BT during the course of experimental rat pancreatitis.

MATERIALS AND METHODS

The study was approved by the Institutional Animal Use and Care Committee of the Gulhane Medical Academy and performed in accordance with the National Institutes of Health guidelines for the care and handling of animals.

Animals

Eighty-five male Sprague-Dawley rats weighing from 280 to 350 g were obtained from Gulhane School of Medicine Research Center (Ankara, Turkey). Before the experiment, the animals were fed standard rat chow and water ad libitum and housed in metabolic cages with controlled temperature and 12 h light/dark cycles for at least 1 wk.

Induction of pancreatitis

Anesthesia was induced with Sevoflurane (Sevorane® Liquid 250 mL, Abbott, Istanbul, Turkey) inhalation. Laparotomy was performed through a midline incision. The common biliopancreatic duct was cannulated with a 28 gauge 1/2 inch, micro-fine catheter. One microaneurysm clip was placed on the bile duct below the liver and another around the common biliopancreatic duct at its entry into the duodenum to avoid reflux of enteric contents into the duct. One mL/kg of 3% sodium taurocholate (Sigma, St. Louis, MO, USA) was slowly infused into the common biliopancreatic duct, with the infusion pressure maintained below 30 mmHg, as measured with a mercury manometer[15]. When the infusion was finished, the microclips were removed, and the abdomen was closed in two layers. All procedures were performed using sterile techniques.

Study protocol

Fifteen rats had a sham operation and served as the controls (Group Ⅰ). AP was induced *via* intraductal taurocholate infusion in the remaining seventy rats. Five of seventy rats died during the 6 h induction period. All surviving animals were randomization into four groups, six hours after the induction of pancreatitis. Group Ⅱ (n = 17) received saline (1 mL/kg, bid, sc), Group Ⅲ (n = 16) ALPL (200 mg/kg per day, bid, sc)[16], Group Ⅳ (n = 16) ALPL plus HBO (2.8 atmospheric pressure, bid, 90 min each, total 4 sessions)[17] and Group Ⅴ (n = 16) HBO alone. Five rats in Group Ⅱ, three rats each in Groups Ⅲ and Group Ⅳ, and four rats in Group Ⅴ died during the treatment period. Fifty-four hours after induction, all the surviving animals were killed with an intracardiac injection of pentobarbital (200 mg/kg). Data was collected on serum amylase levels, oxidative stress parameters [MDA, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx)], bacterial translocation and histopathologic scores.

Laboratory tests

Blood samples were taken from the heart before the animals were sacrificed for serum amylase levels. A Hitachi 917 auto analyzer (Roche Diagnostics, Germany) was used for the amylase assay. Amylase level was expressed as U/L.

Histopathologic analysis

A portion of the pancreatic tissue from each rat was fixed in 10% neutral buffered formalin and embedded in paraffin. One paraffin section, stained with hematoxylin and eosin, was examined from each animal. Two pathologists who were blinded to the treatment protocol scored the tissues for edema, acinar necrosis, inflammatory infiltrate, hemorrhage, fat necrosis, and perivascular inflammation, in 20 different fields. The scores for each of the histologic abnormalities were added up, with a maximum score of 24, as defined by Schmidt *et al*[18].

Quantitative cultures and bacterial identification

The areas of the pancreas showing macroscopic necrosis and visible mesenteric lymph nodes were excised, weighed, and homogenized. The homogenates were diluted serially, quantitatively plated in duplicate on phenylethyl alcohol and MacConkey Ⅱ agar, and incubated aerobically at 37℃ for 24 h. The bacterial counts were expressed as colony-forming units (cfu/g tissue), and counts of 1000 cfu/g or higher were considered to be indicative of a positive culture. Gram-negative bacteria were identified with the API-20E system (BioMerieux Vitek, Hazelwood, MO, USA). Gram-positive bacteria were identified to the genus level by means of standard microbiologic methods[19,20].

Evaluation of oxidative stress

Pancreatic tissue samples were homogenized in cold KCl solution (1.5%) in a glass homogenizer on ice. The samples were centrifuged and the supernatant was used for the assays described below.

Tissue MDA concentration was estimated by the method of Ohkawa *et al*[21]. The supernatant was

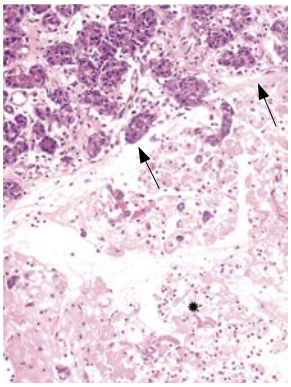

Figure 1 Light microscopy showing extensive necrosis (star) and relatively normal acinar structure (arrows) between the necrotic areas in the control group (HE, × 200).

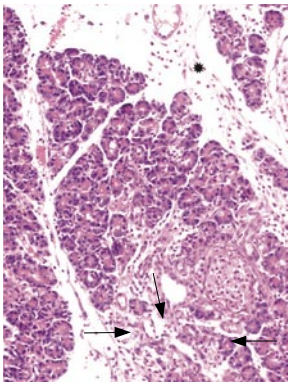

Figure 2 Light microscopy showing interlobular edema (star) and necrotic area (arrows) in the combined treatment group (HE, × 200).

resuspended in 4 mL water, 0.5 mL glacial acetic acid and 0.5 mL 0.33% aqueous thiobarbituric acid solution. The mixture was heated for 60 min in a boiling water bath. After cooling, the complex formed by thiobarbituric acid reactant substances was extracted into an n-butanol phase, and the formed chromogen was measured at 532 nm by spectrophotometer. A standard absorption curve for MDA was prepared using tetramethoxy propane solution. MDA levels were expressed as nmol/g tissue.

For the measurement of SOD activity, the supernatant was diluted 1:400 with 10 mmol/L phosphate buffer, pH 7.00. Twenty five μL of diluted supernatant was mixed with 850 μL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) in a buffer solution containing 50mmol/L 3-cyclohexylaminol-1-propanesulfonic acid (CAPS) and 0.94 mmol/L ethylenediamine tetraacetic acid (EDTA) at pH 10.2. At this stage, 125 μL of xanthine oxidase (80 U/L) was added to the mixture and the absorbance increase was followed at 505 nm for 3 min against air. Twenty five microlitres of phosphate buffer or 25 μL of various standard concentrations were used as blank or standard determinations. SOD activity was expressed as U/g tissue[22].

For GSHPx measurement, the reaction mixture consisted of 50 mmol/L tris buffer, pH 7.6 containing 1mmol/L of Na_2EDTA, 2 mmol/L of reduced glutathione (GSH), 0.2 mmol/L of reduced nicotinamide adenine dinucleotide (NADPH), 4 mmol/L of sodium azide and 1000 U of glutathione reductase (GR), Fifty microlitres of supernatant and 950 μL of reaction mixture, or 20 μL of supernatant and 980 μL of reaction mixture were mixed and incubated for 5 min at 37℃. The reaction was initiated with 8 mmol/L H_2O_2, and the decrease in NADPH absorbance was followed at 340 nm for 3 min. The enzyme activity was expressed as U/g tissue[23].

Table 1 Serum amylase levels, oxidative stress parameters, bacterial translocation, histopathologic scores, and mortality rates in the different study groups

	Group Ⅰ (n = 15)	Group Ⅱ (n = 12)	Group Ⅲ (n = 13)	Group Ⅳ (n = 13)	Group Ⅴ (n = 12)
Amylase (U/L)	278 ± 44	1664 ± 234	974 ± 110	384 ± 40	851 ± 56
Oxidative stress					
MDA (nmol/g)	12.3 ± 0.4	28.3 ± 0.7	18.2 ± 0.4	16.4 ± 0.4	20.1 ± 0.5
SOD (U/g)	395 ± 7	254 ± 6	345 ± 16	282 ± 8	300 ± 9
GSHPx (U/g)	51.6 ± 2.0	30.8 ± 0.9	35.8 ± 1.5	48.4 ± 0.7	45.6 ± 1.4
Bacterial translocation					
MLNs	2 (13%)	12 (100%)	8 (62%)	6 (46%)	7 (58%)
Pancreas	2 (13%)	12 (100%)	7 (54%)	3 (23%)	6 (50%)
Histopathologic score	2 (1-3)	18 (14.5-19)	13 (12.5-15)	6 (4.5-7.5)	9.5(7-11.75)
Mortality	0/15 (0%)	5/17 (29%)	3/16 (19%)	3/16 (19%)	4/16 (25%)

MDA: Malondialdehyde; SOD: Superoxide dismutase; GSHPx: Glutathione peroxidase; MLNs: Mesenteric lymph nodes.

Statistical analysis

The results of parametric tests were expressed as mean ± SE. Nonparametric values were expressed as median (25-75 percentiles). The significance of differences in the histopathologic scores and serum amylase levels was assessed by the Kruskal-Wallis test. Subgroup analyses were performed by the Mann-Whitney *U* test or *t*-test as appropriate. The significance of differences in oxidative stress parameters was determined by Oneway ANOVA test and Tukey HSD procedure as post hoc test. Probabilities less than 0.05 were considered significant. All statistical measurements were made using SPSS PC ver. 11.05 (SPSS Inc. USA).

RESULTS

All rats except those in Group I developed acute pancreatitis, demonstrated by macroscopic parenchymal necrosis, and abundant turbid peritoneal fluid (Figure 1). Histopathological scores were significantly lower in all treatment groups (Group Ⅲ, Group Ⅳ and Group Ⅴ) compared to Group Ⅱ (13 [12.5-15], 6 [4.5-7.5], 9.5 [7-11.75], 18 [14.5-19]; $P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively). The most favorable results were seen in the combination treatment group (Figure 2, Table 1).

Serum amylase levels were lower in Groups Ⅲ, Ⅳ and Ⅴ compared to Group Ⅱ (974 ± 110, 384 ± 40, 851 ± 56, 1664 ± 234 U/L; $P < 0.05$, $P < 0.001$, and $P < 0.02$, respectively, Table 1). Oxidative stress markers showed significantly lower levels in all treatment groups compared to the controls. Tissue MDA levels in Groups Ⅲ, Ⅳ, and Ⅴ were significantly lower than in Group Ⅱ (18.2 ± 0.4, 16.4 ± 0.4, 20.1 ± 0.5 nmol/g, *vs* 28.3 ± 0.7 nmol/g, respectively,

$P < 0.01$ for all, Table 1). Tissue SOD activity in Groups Ⅲ, Ⅳ, and Ⅴ was significantly higher compared to Group Ⅱ (345 ± 16 U/g, 282 ± 8 U/g, 300 ± 9 U/g, *vs* 254 ± 6 U/g; respectively, $P < 0.01$ for all, Table 1). In addition, GSHPx activity in Groups Ⅲ, Ⅳ and Ⅴ was significantly higher than in Group Ⅱ (35.8 ± 1.5 U/g, 48.4 ± 0.7 U/g, 45.6 ± 1.4 U/g, *vs* 30.8 ± 0.9 U/g; respectively, $P < 0.01$ for all, Table 1). BT to pancreas and mesenteric lymph nodes was reduced significantly in the three treatment groups (Group Ⅲ, Ⅳ and Ⅴ) compared to the control group (pancreatic tissue: 54%, 23%, 50%, *vs* 100%; $P < 0.02$, $P < 0.001$, and $P < 0.002$, respectively; MLNs: 62%, 46%, 58%, *vs* 100%; $P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively, Table 1). Bacterial growth was seen in all tissue specimens obtained from the pancreas and MLNs in the control groups. Of the three treatment groups, combination treatment (Group Ⅳ) was most effective in preventing BT (3/13 [23%] to pancreatic tissue, and 6/13 [46%] in MLNs). The best results in terms of amylase levels, histopathological score, oxidative stress markers and BT were seen in rats receiving the combination treatment, compared to animals receiving a single treatment and the control group. Five rats in Group Ⅱ, three rats each in Groups Ⅲ and Ⅳ, and four rats in Group Ⅴ died before the 54th h of induction of pancreatitis. Mortality rates between groups, except the sham group, were statistically not significant.

DISCUSSION

Pancreatic infection is a serious complication of acute necrotizing pancreatitis. The failure of gut barrier results in bacterial translocation and subsequently septic complication of pancreatitis[2,3,24]. For this reason, prevention of contamination of the necrotic pancreatic tissue is very important, and the new generation antibiotics are of significant advantage in this respect. Although in experimental and clinical studies, the use of antibiotic has been shown to be beneficial[2]; in a randomized, controlled study prophylactic antibiotic therapy was found to have no effect on the mortality[25].

Although the potential role of xanthine oxidase in the presence of barrier failure and translocation of bacteria across the gut lumen has been shown in a previous study[14], the degree to which such a mechanism is involved in the pathogenesis of pancreatic infection is not known, and whether an inhibitor of this enzyme has a preventive effect is not clear[11,12]. The role of HBO therapy in the prevention of infectious complications, mainly through the reduction of oxidative stress and bacterial translocation in experimental acute pancreatitis has been reported previously[7,26]. Our group had previously investigated the efficacy of individual administration of allopurinol and HBO in preventing bacterial contamination of pancreatic tissue. In the present study, we examined the impact of combining allopurinol and HBO therapy[7,14].

We observed that both allopurinol and HBO had beneficial effects on the biochemical and histological abnormalities, oxidative stress and bacterial translocation. The present report represents the first study examining the effects of a xanthine oxidase inhibitor plus HBO therapy in acute pancreatitis. The individual effects of the two treatments on amylase levels were nearly the same. However, HBO treatment resulted in greater reduction in the histopathological scores, while allopurinol alone did not produce satisfactory histological recovery. The histological abnormalities in the combined treatment group were significantly less compared with the use of allopurinol and HBO alone, indicating a potentiation of effect. Allopurinol also decreased the oxidative stress parameters, as it has been reported previously[13,14,27,28], although allopurinol was found to have no effect on the incidence and severity of endoscopic retrograde cholangiopancreatography (ERCP)-induced pancreatitis in studies on human subjects by Budzynska *et al*[29]. When allopurinol was co-administered with HBO at the same doses, the overall antioxidant effect did not increase. These results correlate well with the histological recovery seen in animals treated with individual drugs and combination therapy. However, when the data regarding oxidative stress was examined, it was interesting to note that combination therapy was more effective in increasing the anti-oxidant system. These findings suggest that the improvement in pancreatic morphology was related to the increase in the anti-oxidant system.

Bacterial translocation was very similar in the individual treatment groups. Again, bacterial contamination of the pancreatic tissue was significantly less in the combined treatment group, indicating a potentiating effect. Although xanthine oxidase, an important source of endothelial cell-derived superoxide and hydrogen peroxide, plays a primary role in ischemia-reperfusion injury, which contributes to the failure of the intestinal barrier[11], it can be postulated that even with the addition of antioxidant activity of allopurinol, inhibition of xanthine oxidase was not superior compared to the use of HBO alone. However, a combination of these two agents may produce remarkable inhibition of bacterial translocation, perhaps through different mechanisms including not only xanthine oxidase inhibition and antioxidant activity but also direct antibacterial, immunological, angiogenic and cellular-subcellular effects.

Finally, the present study confirmed our previous observations on the efficacy of HBO and allopurinol in experimental acute necrotizing pancreatitis and also demonstrated that a combination of these treatment options prevented more effectively the progression of pancreatic injury. Nevertheless, the activity and potency of xanthine oxidase, the importance of blocking its activity, and the detailed effects of HBO on this enzyme in the intestines and in the pancreas in acute pancreatitis need further examination.

COMMENTS

Background

The severity of acute pancreatitis may range from a mild, self-limited illness to a catastrophic disease with multiple potentially severe complications and risk of death. Translocation of bacteria from the intestines is one of the most important factors in the development of septic complications and mortality in acute pancreatitis

Research frontiers

Most of the experimental and clinical studies designed to reduce morbidity and mortality in acute pancreatitis are focused on minimizing the extent of necrosis and the prevention of bacterial contamination of necrotic pancreatic tissue.

Innovations and breakthroughs

Several studies have assessed the effect of allopurinol and hyperbaric oxygen on bacterial translocation, oxidative stress, and histology in experimental acute necrotizing pancreatitis. The present study was carried out a rat model to evaluate the effect of combined allopurinol and hyperbaric oxygen treatment on bacterial translocation, oxidative stress and the course of acute necrotizing pancreatitis.

Applications

If these results are confirmed on further studies, combination treatment with allopurinol and hyperbaric oxygen can be applied clinically in patients with acute necrotizing pancreatitis to prevent oxidative stress and bacterial translocation.

Peer review

This paper examines the effects of allopurinol and hyperbaric oxygen on taurocholate infusion-induced acute necrotic pancreatitis in rats. It was observed that both treatments improved the pathological abnormalities, and combination of the two modalities provided further improvement.

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RAPID COMMUNICATION

Early diagnosis and prediction of severity in acute pancreatitis using the urine trypsinogen-2 dipstick test: A prospective study

Erdinc Kamer, Haluk Recai Unalp, Hayrullah Derici, Tugrul Tansug, Mehmet Ali Onal

Erdinc Kamer, Haluk Recai Unalp, Hayrullah Derici, Tugrul Tansug, Mehmet Ali Onal, Department of Surgery, Izmir Ataturk Training and Research Hospital, Izmir 35820, Turkey
Correspondence to: Dr. Erdinc Kamer, Department of Surgery, Izmir Ataturk Training and Research Hospital, 1834 sk. No. 9/4 35530 Karşıyaka- İzmir, Turkey. erdinc.kamer@gmail.com
Telephone: +90-232-2444444 Fax: +90-232-2434848
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Abstract

AIM: To evaluate the use of the trypsinogen-2 dipstick (Actim Pancreatitis) test for early diagnosis and prediction of severity in acute pancreatitis (AP).

METHODS: Ninety-two patients with AP were included in this study. The control group was 25 patients who had acute abdominal pain from non-pancreatic causes. Urine trypsinogen-2 dipstick test (UTDT) and conventional diagnostic tests were performed in all patients. Patients were divided by the Atlanta classification into two groups as having mild or severe pancreatitis.

RESULTS: UTDT was positive in 87 (94.6%) of the AP patients and in two (8%) controls ($P < 0.05$). Positive UTDT was found in 61 (92.4%) of 66 (71.7%) patients with mild pancreatitis and in all (100%) of the 26 (28.3%) with severe pancreatitis ($P > 0.05$). UTDT positivity lasted longer in severe pancreatitis compared with that in mild pancreatitis (6.2 ± 2.5 d *vs* 2.0 ± 1.43 d, $P < 0.05$). The sensitivity, specificity, positive predictive value, negative predictive value (NPV), positive likelihood ratio (PLR) and negative likelihood ratio (NLR) of UTDT were 91%, 72%, 96.6%, 70.4%, 3.4 and 0.1, respectively.

CONCLUSION: UTDT is a simple, rapid and reliable method for use on admission. It has high specificity and low NLR for early diagnosis and prediction of severity in AP. However, its relatively low NPV does not allow trypsinogen-2 dipstick test to be a stand-alone tool for diagnosis of acute pancreatitis; the use of other conventional diagnostic tools remains a requirement.

Key words: Acute pancreatitis; Urine trypsinogen-2 dipstick test; Early diagnosis; Disease severity

INTRODUCTION

Most patients with acute pancreatitis (AP) have a mild and self-limited form of the disease that resolves spontaneously, but approximately 20% of attacks are severe and represented by pancreatic necrosis, sepsis, and fulminant multiorgan/system failure with a life-threatening morbidity and a mortality rate of 20%-30%. Hence, early diagnosis and prediction of severity in AP has particular significance[1-6].

Early prediction of the severity of AP is difficult[3,7]. Multifactorial scoring systems like the Ranson prognostic signs and the Glasgow score can only be evaluated 48 h after admission. The acute physiology and chronic health evaluation Ⅱ (APACHE Ⅱ) score has the invaluable advantage of being useful within a few hours after admission, and it can be assessed serially. However, it is cumbersome, which limits its use in clinical practice[7]. The current gold standard for staging AP combines clinical criteria with computed tomography (CT), but this has limited availability, high costs, exposes the patient to ionizing radiation, and lacks sensitivity and specificity in the early stage of the disease. Several laboratory markers have been evaluated as replacements for the multifactorial scoring systems, with CT being the most widely used[8,9]. Alternatively, magnetic resonance imaging can be used, e.g. in case of contraindications to intravenous CT contrast agents[10]. Various biochemical tests such as the urine trypsinogen-2 dipstick test (UTDT) have been developed over the past ten years for early diagnosis and prediction of severity in AP. Trypsinogen occurs as two major isoenzymes, trypsinogen-1 (cationic) and trypsinogen-2 (anionic), which are secreted at high concentrations into pancreatic fluid with a small proportion escaping into the circulation. Trypsinogen-1 and trypsinogen-2 are eliminated from the blood circulation by the kidneys. In AP, concentrations of trypsinogen-2 in serum and urine are higher than those of trypsinogen-1[11-13]. Although

UTDT has been evaluated in many studies, details of the clinical use of this test for early diagnosis and prediction of severity in AP remain obscure[3,13-15]. The aim of our prospective study was to evaluate the use of the trypsinogen-2 dipstick (Actim Pancreatitis) test in early diagnosis and prediction of severity in AP, and to compare the sensitivity, specificity and prognostic value of the this test with those of serum amylase, serum lipase and APACHE Ⅱ score.

MATERIALS AND METHODS

Materials

The prospective study population consisted of 92 consecutive patients with AP (study group: 69 males, 23 females; median age 58.9 year, range 36-80) and 25 consecutive patients with acute abdominal disease of extrapancreatic origin (control group: 15 males, 10 females; median age 59.2 year, range 34-78) admitted to the emergency unit at Izmir Ataturk Education and Research Hospital between January 2003 and July 2005. The study was approved by the Committee on Research Ethics at our hospital, and all patients gave their informed consent for inclusion in the study.

Study design

Diagnosis of AP was based on a history of prolonged upper abdominal pain, serum amylase at least three times the upper limit of normal and the presence of edema or necrosis on abdominal ultrasonography and/or contrast-enhanced CT. Patients who were admitted after the first 24 h after the onset of abdominal pain were not included in the study. APACHE Ⅱ score values were calculated on admission and at 48 h. Body mass index (BMI) of all patients was calculated. CT was performed selectively in patients with severe pancreatitis as predicted by either one of the two scoring systems (Ranson criteria > 2 or APACHE Ⅱ score > 7). Under the Atlanta classification, AP is predicted as severe if it is accompanied by single or multiorgan failure, local complications, 3 or more on the Ranson criteria, or an APACHE Ⅱ score of ⩾ 8 points[1].

Methods

Serum amylase and lipase concentrations were measured using enzymatic assay (Architect C 8000; Abbott, Abbott Park, IL, USA; reference interval, 26-100 U/L and 13-60 U/L, respectively). The Actim Pancreatitis test strip (Medix Biochemica, Kauniainen, Finland), an immunochromatographic test, was used for urine trypsinogen-2 determination (detection limit 50 μg/L). The tip of the strip was immersed into a urine-containing vial and was held for 20 s before being completely taken out of the vial. The strip was then kept at room temperature for 5 min to observe whether urine reacted with blue latex particles covered by monoclonal antitrypsinogen-2 antibodies. Excess (> 50 μg/L) urinary trypsinogen-2 caused the occurence of 2 blue stripes, while only one stripe (referred to as the control stripe) was observed when urinary trypsinogen-2 concentration was within the normal range. The appearance of the control stripe confirmed the accuracy of the assay, while no blue stripes on the test strip suggested an erroneous test, in which case the test was repeated[16].

Table 1 Diagnosis and/or etiology of pancreatitis in the control and study groups

Control group	*n* (%)	Study group	*n* (%)
Familial Mediterranean fever	5 (20)	Gallstone	58 (63.0)
Acute appendicitis	5 (20)	Idiopatic	18 (19.6)
Acute cholecystitis	4 (16)	Post-ERCP	8 (8.7)
Perforated peptic ulcer	3 (12)	Alcohol	5 (5.4)
Acute cholangitis	2 (8)	Hypertriglyceridemia	3 (3.3)
Pelvic inflammatory disease	2 (8)		
Intestinal obstruction	2 (8)		
Gastric cancer	1 (4)		
Meckel diverticulitis	1 (4)		

ERCP: Endoscopic retrograde cholangiopancreaticography.

Statistical analysis

Data were expressed as the mean ± SE. The Mann-Whitney *U* and McNemar tests were used where appropriate for statistical analysis. All *P* values were two-tailed, and those with $P < 0.05$ were defined as statistically significant. For serum concentrations of amylase and lipase, a threefold increase in the reference values recommended by our laboratory were selected as cut-off values. Using these cut-off points, the sensitivity, specificity, positive (PPV) and negative predictive value (NPV), and positive (PLR) and negative likelihood ratio (NLR) in establishing the diagnosis of AP were calculated. The SPSS/PC 10.0 (SPSS, Chicago, IL, USA) statistical package was used on a personal computer for the analysis of data.

RESULTS

The mean ages of patients in the study and control groups were 58.9 ± 14.2 year ($n = 92$, range, 36-80) and 59.2 ± 13.5 year ($n = 25$, range, 34-78), respectively ($P = 0.928$). No significant difference was found between the two groups in terms of gender (study group, M/F = 69/23; control group, M/F = 15/10; $P = 0.113$). Gallstones were the most common causes of AP ($n = 58$, 63%), while familial Mediterranean fever and acute appendicitis were the most common causes of acute abdominal pain in patients in the control group ($n = 5$, 20% and $n = 5$, 20%, respectively) (Table 1). Mean BMI of patients in the study and control groups were 24.0 ± 3.8 kg/m^2 (range, 16-33) and 23.5 ± 3.4 kg/m^2 (range, 16-30), respectively ($P = 0.902$). Both mean serum amylase concentrations and mean serum lipase concentrations were significantly higher in patients in the study group compared with those in the control [amylase, 600.8 ± 189.7 U/L (range, 376-1250) *vs* 67 ± 32.5 U/L (range, 18-176), and lipase, 96.2 ± 42.4 U/L (range, 58-230) *vs* 33.8 ± 15 U/L (range, 13-65); $P = 0.000$)].

UTDT on admission was positive in 87 (93.5%) of the 92 patients in the study group, and in two (8%) of the 25 patients in the control group ($P = 0.000$). In the control

Table 2 Demographics and results for control and study groups

	Control group (n = 25)	Study group (n = 92)	P
Age (yr)[1]	59.2 ± 13.5 (34-78)	58.9 ± 14.2 (36-80)	0.928
Gender (F/M)	15/10	69/23	0.113
BMI (kg/m^2)[1]	23.5 ± 3.4 (16-30)	24.0 ± 3.8 (16-33)	0.902
Amylase (> 100 U/L)[1]	67 ± 32.5 (18-176)	600.8 ± 189.7 (376-1250)	0.000
Lipase (> 60 U/L)[1]	33.8 ± 15 (13-65)	96.2 ± 42.4 (58-230)	0.000
UTDT +/- (on admission)	2/23	87/5	0.000
UTDT +/- (48 h later)	1/23	87/5	0.000
Duration of UTDT positivity (d)[1]	2.0 ± 1.4 (1-3)	3.6 ± 2.1 (2-13)	0.000
APACHE Ⅱ score (on admission)[1]	4.6 ± 1.5 (3-8)	6.1 ± 2.7 (3-13)	0.006

[1]Values expressed in mean ± SE (inter-quartile range).

Table 3 Demographics and results for control and study groups (Classified as mild and severe AP)

	Control (n = 25)	Mild AP (n = 66)	Severe AP (n = 26)	P^1	P^2	P^3
Age (yr)[4]	59.2 ± 13.5	58.7 ± 14.3	60.2 ± 14.9	0.883	0.796	0.655
Gender (F/M)	15/10	51/15	17/9	0.118	0.776	0.365
BMI (kg/m^2)[4]	23.5 ± 3.4	23.5 ± 3.9	24.2 ± 4.1	0.627	0.507	0.217
Amylase (> 100 U/L)[4]	67 ± 32.5	578.6 ± 171	658.2 ± 222	0.000	0.000	0.068
Lipase (> 60 U/L)[4]	33.8 ± 1	76.0 ± 23.9	110.4 ± 54.2	0.000	0.000	0.000
Positive UTDT (on admission)	2/23	61/5	26/0	0.000	0.000	0.351
Positive UTDT (48 h later)	1/24	61/5	26/0	0.000	0.003	0.351
Duration of UTDT positivity (d)[4]	2.0 ± 1.4	2.6 ± 0.6	6.2 ± 2.5	0.000	0.000	0.000
APACHE-Ⅱ score (on admission)[4]	4.6 ± 1.5	4.7 ± 1.4	9.7 ± 1.8	0.807	0.000	0.000
APACHE-Ⅱ score (after 48 h)[4]	-	4.24 ± 1.4	11.5 ± 2.9	-	-	0.000
Ranson score (on admission) > 2[4]	-	1.03 ± 0.8	3.73 ± 1	-	-	0.000

[1]Control group *vs* mild AP; [2]Control group *vs* severe AP; [3]Mild AP *vs* severe AP; [4]Values expressed as means ± SD.

group, false-positive UTDTs were normalized 1 d later in a patient with acute cholecystitis and 3 d later in a patient with gastric cancer. On the other hand, UTDT positivity lasted for an average of 3.6 ± 2.1 d (range, 2-13) in patients in the study group. APACHE Ⅱ score (cut-off > 8) was 6.1 ± 2.7 (range, 3-13) and 4.6 ± 1.5 (range, 3-8) in the study and control groups, respectively (P = 0.006) (Table 2).

Of the patients with AP, 66 (71.7%) had mild disease and 26 (28.3%) had severe disease according to the Atlanta classification[1]. Gallstones were the most common cause of both mild and severe AP (n = 45, 68.2% and n = 13, 50%, respectively). No significant difference was found between patients with mild and severe AP in terms of BMI (P = 0.217). However, both Ranson and APACHE Ⅱ scores on admission were significantly higher in patients with severe AP than in those with mild AP [Ranson, 3.73 ± 1.04 (range, 2-5) *vs* 1.03 ± 0.82 (range, 0-3), and APACHE Ⅱ, 9.73 ± 1.75 (range, 8-13) *vs* 4.68 ± 1.38 (range, 3-8), respectively; P = 0.000]. Likewise, APACHE Ⅱ score determined at 48 h later was significantly higher in patients with severe pancreatitis than in those with mild pancreatitis (4.24 ± 1.44 *vs* 11.5 ± 2.88, P = 0.000). Sensitivity and specificity of APACHE Ⅱ score on admission were 56.0% and 89.4%, respectively.

Table 4 Sensitivity, specificity, PPV, NPV, PLR and NLR of serum amylase, serum lipase, UTDT and APACHE Ⅱ scoring systems in AP

	Sensitivity %	Specificity %	PPV %	NPV %	PLR	NLR
On admission						
Serum amylase (> 100 U/L)	78.0	87.3	94.8	61.5	6.1	0.3
Serum lipase (> 60 U/L)	86.2	89.4	96.6	76.0	8.1	0.2
UTDT, positive	91.0	72.0	96.6	70.4	3.4	0.1
APACHE Ⅱ > 8	56.0	89.4	61.0	84.2	5.3	0.5

Table 5 Comparisons of sensitivity and specificity of UTDT in AP in present study with those reported previously

Reference	Sensitivity (%)	Specificity (%)
Kemppainen *et al* 1997[29]	94	95
Kylanpaa-Back *et al* 2000[28]	96	92
Lempinen *et al* 2001[5]	62	87
Pezzilli *et al* 2001[27]	53.3	-
Lempinen *et al* 2003[7]	72	81
Chen *et al* 2005[16]	89.6	85.7
Saes *et al* 2005[30]	68	86.4
Present study	91	72

Mean serum amylase concentrations were not significantly different in patients with severe and mild AP (658.2 ± 222.5 U/L *vs* 578.6 ± 171.3 U/L, respectively, P = 0.068), while serum lipase concentrations in patients with severe AP were significantly higher than those in patients with mild AP (76.0 ± 23.9 U/L *vs* 110.4 ± 54.2 U/L, respectively, P = 0.000). UTDT was positive in 61 of the 66 (92.4%) patients with mild AP and in all 26 (100%) patients with severe AP (P = 0.351). No significant difference was found between patients with severe AP and those with mild AP in terms of UTDT positivity at 48 h after admission (P = 0.351). Positive UTDT continuation averaged 2.6 ± 0.6 d (range, 2-4) and 6.2 ± 2.5 d (range, 3-13) in patients with mild and severe AP, respectively (P = 0.000) (Table 3).

In AP, the sensitivity, specificity, PPV, NPV and PLR of serum amylase and lipase were 78%, 87.3%, 94.8%, 61.5% and 6.1, and 86.2%, 89.4%, 96.6%, 76.0% and 8.1 respectively, while UTDT sensitivity was 91%; specificity was 72%; PPV was 96.6%; NPV was 70.4% and PLR was 3.4 (Table 4). Sensitivity and specificity rates of UTDT obtained in our study were compared with those reported in the literature and are listed in Table 5.

DISCUSSION

AP presents in various clinical forms ranging from

mild abdominal discomfort to multiple organ failure. After a mild pancreatitis attack, 80% of patients recover completely, while the disease worsens in 20% and has a mortality rate of 30%[1,17-19]. Thus, early diagnosis and determination of severity of AP are of great importance in terms of mortality and morbidity.

Several methods have been used to diagnose AP and determine its prognosis and severity; these include scoring systems (such as Ranson, Glasgow, and APACHE), biochemical parameters [e.g. serum amylase, lipase, C-reactive protein (CRP), trypsinogen-activation peptide (TAP), interleukins 6 and 8, carboxypeptidase B activation peptide, tumor necrosis factor-α, platelet-activating factor, polymorphonuclear elastase, and serum procalcitonin], and imaging techniques (such as CT)[5,20-22]. The methods that can be used to compare the predictive value of different tests have been summarized in a paper by Jaeschke[23]. What is needed is an immediate test with high specificity and low NLR[24].

In the mid 1990s, urine trypsinogen concentration and TAP were reported to be of high sensitivity and specificity in diagnosing and predicting severity of AP. Since then, determinations of urine trypsinogen concentration and TAP have been considered as good alternative biochemical tests[25,26]. Lempinen et al have compared urinary trypsinogen-2 with urinary TAP and serum CRP for early differentiation between severe and mild AP and concluded that urinary trypsinogen-2 is superior to serum CRP, and is as good as or even better than urinary TAP for the early prediction of disease severity in the first 24 h of admission for AP[7].They have also noted that the result of a trypsinogen-2 dipstick test is available within 5 min, whereas TAP requires a laborious ELISA method, which takes several hours and requires skilled laboratory personnel; the rapid urinary trypsinogen-2 test does not require the use of laboratory equipment[7].

Sensitivity and specificity of UTDT in AP has been reported in the literature as 53.3%-96% and 85.7%-95%, respectively[22,25-31]. We calculated a sensitivity of 91% and specificity of 72% for UTDT. Consistent with a previous report, we found higher sensitivity for UTDT compared with that for serum amylase and lipase concentrations (91% *vs* 78% and 86%, respectively)[4]. However, Pezzilli *et al* reported a low sensitivity for UTDT in their study in which 30 patients with AP were investigated, 11 of whom were included at 2-3 d after onset of the attack[27]. We believe that this late inclusion of a considerable number of patients in the aforementioned study might have affected urinary trypsinogen-2 concentrations and, thus might have decreased the sensitivity and specificity of UTDT for AP diagnosis. In agreement with this view, Chen et al have recently reported a gradually decreasing sensitivity for UTDT in diagnosis of AP from the first to the fourth day of admission (i.e. 90.6%, 81.2%, 59.4% and 50% on the first, second, third and fourth days of admission, respectively)[16]. Considering the effect of late admission (which resulted in delayed UTDT), we did not include patients who were admitted 24 h after the onset of abdominal pain. Thus, we obtained a homogeneous study group in terms of timing of UTDT. We found a positive UTDT in 93.5% and 8% of patients with AP and those with acute abdominal pain due to non-pancreatic causes, respectively. This statistically significant difference supports the use of UTDT in AP diagnosis within the first 24 h of acute abdominal pain.

Chen *et al* have concluded that UTDT can be used in the differential diagnosis of AP due to its high NPV[16]. Lempinen *et al* and Kylanpaa-Back *et al* have reported NPV rates for UTDT of 85% and 99%, respectively[5,28]. We found that the NPV for UTDT was 70.4%, which is higher than the NPV of serum amylase and close to that of serum lipase. We believe that AP diagnosis must be confirmed by other biochemical tests and imaging techniques in patients with a positive UTDT. This is because urinary trypsinogen-2 concentrations may also be increased in other diseases such as hepatobiliary and pancreatic malignancies, colon cancers, and chronic pancreatitis[13]. On the other hand, we found that the PPV (96.6%) for UTDT was higher than that of serum amylase and was equal to that of serum lipase. Thus, we believe that the use of UTDT is advantageous for an early diagnosis of AP because of its rapid action, high sensitivity and high PPV.

UTDT has been reported to have a direct correlation with the severity of AP, as its sensitivity increases with increased severity of AP[4,16]. We have been unable to confirm this conclusion, because in our study we found the sensitivity of UTDT was 87.2% in severe AP and 82.8% in mild AP. However, these rates were not significantly different. On the other hand, a longer duration of UTDT positivity in severe AP compared with that in mild AP was detected. These data suggest that repeating UTDT every day may allow a clinician to predict the severity of AP that varies with time. Thus, such patients will probably benefit from admission to a medical center, prophylactic antibiotic administration, early enteral nutrition, and early endoscopic retrograde cholangiopancreatography in pancreatitis of suspected biliary origin[25].

In conclusion, while there are a few studies on the value of the trypsinogen-2 dipstick test as a predictive test for the severity of AP, its relatively low NPV does not allow UTDT to be a stand-alone tool for diagnosis of AP. Thus, the use of other conventional diagnostic tools becomes an additional requirement. However, UTDT is a simple, rapid and a reliable method that can be used on admission with high specificity and low NLR for early diagnosis and prediction of severity in AP.

COMMENTS

Background

Urine trypsinogen-2 dipstick test (UTDT) is simple, rapid and a reliable method that can be used on admission with high specificity and low negative likelihood ratio (NLR) for early diagnosis and prediction of severity in acute pancreatitis (AP).

Research frontiers

Early diagnosis and prediction of severity in AP is of particular significance.

Innovations and breakthroughs

Although UTDT has been evaluated in many studies, clinical use of this test for early diagnosis and prediction of severity in AP is obscure. The aim of our

prospective study was to evaluate the use of a trypsinogen-2 dipstick (Actim Pancreatitis) test in early diagnosis and prediction of severity in AP and to compare the sensitivity, specificity and prognostic value of this test with those of serum amylase, serum lipase and APACHE Ⅱ score.

Applications

UTDT is a simple, rapid and reliable method that can be used on admission for early diagnosis and prediction of severity in AP.

Terminology

Various biochemical tests, one of which is the UTDT, have been developed over the past ten years for early diagnosis and prediction of severity in AP. Trypsinogen occurs as two major isoenzymes, trypsinogen-1 (cationic) and trypsinogen-2 (anionic), which are secreted at high concentrations into pancreatic fluid with a small proportion escaping into the circulation. Trypsinogen-1 and trypsinogen-2 are eliminated from the blood circulation by the kidneys. In AP, concentrations of trypsinogen-2 in serum and urine are higher than those of trypsinogen-1.

Peer review

The authors evaluated the use of a trypsinogen-2 dipstick (Actim Pancreatitis) test in early diagnosis and prediction of severity in AP. UTDT is a simple, rapid and a reliable method that can be used on admission with high specificity and low NLR for early diagnosis and prediction of severity in AP.

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RAPID COMMUNICATION

Effect of Breathwalk on body composition, metabolic and mood state in chronic hepatitis C patients with insulin resistance syndrome

M Vázquez-Vandyck, S Roman, JL Vázquez, L Huacuja, G Khalsa, R Troyo-Sanromán, A Panduro

M Vázquez-Vandyck, S Roman, A Panduro, Molecular Biology in Medicine, Civil Hospital of Guadalajara, Fray Antonio Alcalde, Guadalajara Jalisco, México
M Vázquez-Vandyck, S Roman, A Panduro, Health Sciences University Center, University of Guadalajara, Guadalajara Jalisco, México
JL Vázquez, Epidemiology Department, Regional General Hospital No.110, Mexican Institute of Social Security and Public Health Department, University of Guadalajara, Guadalajara Jalisco, México
L Huacuja, Chronic Degenerative Diseases Institute, Health Sciences University Center, University of Guadalajara, Guadalajara Jalisco, México
G Khalsa, Kundalini Research Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, United States
R Troyo-Sanromán, Research Unit, Health Sciences University Center, University of Guadalajara, Guadalajara Jalisco, México
Supported by Grant Salud-Conacyt-2004-C01-025 to AP
Correspondence to: Arturo Panduro, MD, PhD, Molecular Biology in Medicine, Civil Hospital of Guadalajara Fray Antonio Alcalde, P.O. Box 2-500, Guadalajara, Jalisco 44280, Mexico. apanduro@prodigy.net.mx
Telephone: +52-33-36147743 Fax: +52-33-36147743
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Abstract

AIM: To identify the anthropometric, metabolic and mood state in hepatitis C virus (HCV)-infected patients from the west of Mexico and to evaluate the effect of Breathwalk (BW), a combination of walking, synchronized breathing and focussed attention, on those patients.

METHODS: In an experimental study, 17 patients with serological and molecular diagnosis of HCV, not receiving pharmacological treatment, were studied. One hour sessions of BW were practiced 3 times at week for six months. Body composition was assessed by electric impedance. Biochemical profiles and insulin resistance (IR) risk was assessed by conventional methods. Mood state was evaluated with specific and open questions at the beginning and at the end of the program.

RESULTS: Seventy percent of patients were overweight or obese, and 77% of the patients presented with IR at the beginning of the study. Improvements were observed at the 3rd mo, and statistically significant differences were recorded at the 6th mo using the fitness score (76 *vs* 83, $P < 0.01$), in alanine aminotransferase (ALT) (106 ± 93 U/L *vs* 59 ± 32 U/L, $P < 0.01$), total bilirubin (0.09 ± 1 mg/dL *vs* 0.62 ± 0.2 mg/dL, $P < 0.01$), ALT/AST ratio (1.04 *vs* 0.70, $P < 0.01$), triglycerides (165 ± 86 mg/dL *vs* 124 ± 49 mg/dL, $P < 0.01$) and the IR risk (4.0 *vs* 2.7). Most patients (88%) indicated to feel better at the end of BW ($P < 0.01$).

CONCLUSION: Breathwalk has an important effect on body composition, lipid profile and liver enzymes. It is also easy, inexpensive and has a beneficial effect on metabolic and mood state in HCV patients.

Key words: Breathwalk; Chronic hepatitis C; Insulin resistance; Obesity; Quality of life

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INTRODUCTION

It has been estimated that hepatitis C virus (HCV) has infected more than 170 million people globally and is responsible for up to 70% of all viral chronic infections[1]. Furthermore, obesity, type 2 diabetes (DM2) and insulin resistance (IR) are current health problems that especially affect chronic HCV patients[2-6]. The occurrence of obesity is an additional risk factor that can further deteriorate the health condition of chronic hepatitis C patients[7], with increased insulin resistance[8] and potential development of diabetes and progression to liver fibrosis[9,10]. Also, poor quality of life related to changes in mood, emotional state, and depressions are common findings among chronic HCV patients[11,12]. This is mainly attributed to the impact of the diagnosis and subsequent anxiety over long-term health[12]. This situation may limit the tolerability of antiviral treatment and reduce compliance[11], together with the fact that HCV patients with excess body weight are known to be poor responders to antiviral therapy in comparison to normal-weight individuals[13,14]. Therefore, it is important to search for additional therapeutic strategies

focused on lifestyle habits in chronic HCV patients that can contribute to improve metabolic and liver function as well as better quality of life.

Conventional therapeutic interventions for chronic HCV patients are primarily focused on antiviral regimens with minor attention on lifestyle modifications[15]. For instance, in the past and up to date, it has been stated that patients diagnosed with liver cirrhosis regardless of the stage must remain in a resting state or maintain minimal physical activity. Even when exercise and/or physical activity is a common therapeutic modality recommended for obese, DM2 and IR patients[16,17], studies related to the modality and strategy of exercise that could be beneficial for the patients with chronic liver disease are limited[18]. Breathwalk (BW) is a novel exercise strategy that is different from conventional walking, in fact it synchronizes walking steps with specific breath patterns and mental sustained attention[19].

Since obesity and IR are dependent on genetic and environmental factors of each population, the identification of such variables and implementation of specific strategies are necessary in order to achieve a better quality of life and response to antiviral treatments. The aim of the present study was to measure anthropometric and metabolic parameters as well as the mood state in HCV-infected patients from the west of Mexico and to evaluate the effect of BW on those parameters.

MATERIALS AND METHODS

Patients

In an experimental study, 22 patients with chronic hepatitis C infection attending the Gastroenterology and Molecular Biology in Medicine Departments at the Civil Hospital of Guadalajara were included. Molecular and serological diagnosis of HCV was performed as described before[20]. All patients had serologic and molecular diagnosis (PCR) of chronic hepatitis C virus and were not receiving interferon or any other specific treatment. The Civil Hospital of Guadalajara is one of the biggest public hospitals in Mexico that mainly attends low income patients that have no other social health security. Due to the cost of interferon, the low income level and the large number of patients with HCV attending the hospital, most of them do not receive specific pharmacological treatment. Patients with HCV infection without interferon treatment were invited to participate in the program. Only those volunteers who signed a written consent were included. Clinical evaluations involved identification of the stage of the disease through Child-Pugh score. Patients with decompensated hepatic disease and/or Child C were not included. Twenty two patients remained until after the third month of the program. At this time period, an important improvement was registered, however 5 patients did not continue and dropped out because of the following reasons: two patients were from out of the city and returned to their villages, one patient felt better and decided not to further attend and 2 more patients did not show up. The protocol was conducted in accordance with the Helsinki declaration and with approval by the ethical committee of the hospital.

Body composition

Body composition was measured by electric impedance technique (In Body 3.0, Biospace, Inc). Patients were indicated to fast at least 8 h, to not carry out any type of exercise 5 d before evaluation and to empty the bladder and bowels before testing[21,22]. The parameters evaluated in body composition were body mass index BMI (kg/m^2), percentage of fat and muscle, waist circumference (WC) and fitness score. Patients were classified for risk of metabolic complications associated with obesity, according to WC. High risk of cardiovascular disease was marked at 94 cm of WC for men and 80 cm for women according to WHO[23]. Also the Official Mexican Norm for the classification of BMI[24] was used.

Biochemical profile

Blood samples for measurement of the biochemical profile were obtained after an overnight fast (12 h minimum). The biochemical profile included lipid profile [total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL)], liver function tests aspartate amine transferase (AST), alanine amine transferase (ALT), total bilirubin, glucose and insulin. Routine biochemical test were performed by the use of manual enzymatic assays (Human Gessellschaft für Biochemica und Diagnostica mbH, Germany). Insulin levels were detected by microparticle enzyme immunoassay (Abbott Laboratories, North Chicago, IL). Insulin resistance was measured by the homeostasis model of assessment (HOMA)[25,26] and/or the triglycerides/high-density lipoprotein (TG/HDL) ratio[27].

Evaluation of mood state

Initially and at the end of the BW program, an interview was carried out with specific questions in order to assess the patients' mood regarding their illness during this intervention as described elsewhere[28,29]. Also, an interview was performed with two questionnaires. The first had two types of questions: open and with multiple option answers related to negative and positive mood states. One open question was: How is your mood state actually? Options for positive mood state were: Happy, calm, good in general and negative mood options were: angry, nervous, anguished, fear, bad in general.

The second questionnaire asked specific questions like: "Have you reduced the time spent in your job or other activities?" "Have you been done less of what you like to do?" "Have you done your job or others activities carelessly?" These specific questions had a transformed score after evaluation. Patients were scored in four categories: excellent (100 points), good (66.66 points), regular (33.33) and bad (0 points).

Breathwalk protocol

Breathwalk is an exercise strategy that consists basically of walking with different synchronized breath patterns enhanced with a meditative episode[19]. Physical movements in BW are combined aerobic and resistance exercises. The first and basic tool is a conscious complete deep breathing. While inhaling in a rhythmic and flowing way, air is taken from the stomach and directed smoothly towards the

Table 1 Demographic and clinic characteristics of patients with HCV infection at the beginning of the breathwalking (BW) program

Parameter	HCV group *n* = 17	Reference range
Age (yr)	51 ± 10.2	-
Sex F/M (%)	11/6 (65/35)	-
DM2 (%)	3 (18)	-
BMI (kg/m^2)	27.6 ± 3.2	< 25
Waist circumference (cm)	88.0 ± 10.4	< 80 cm F, < 94 cm M
Fat percentage (mean)	32.3 ± 7.1	< 28% F, < 20% M
Soft lean mass (kg)	44.2 ± 7.9	-
Glucose (mg/dL)	94.0 ± 16.5	< 110
ALT (U/L)	106.7 ± 93.1	< 55
AST (U/L)	101.4 ± 66.6	< 40
Cholesterol (mg/dL)	157.1 ± 35.0	< 200
Triglycerides (mg/dL)	165.0 ± 86.5	< 160
HOMA ratio	3.7 ± 1.8	< 2.5
Metabolic syndrome (%)	10 (59)	[1]

DM2: Type 2 diabetes mellitus; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; F: Female; M: Male; HOMA: Homeostasis model of assessment. [1]NCEP (National cholesterol education program) ATP Ⅲ (adult treatment panel Ⅲ) 2001/2 criteria from metabolic syndrome.

thoracic chest and it finishes in a lightly upward movement of the clavicle and the superior part of the chest. While exhaling, all movements are inverted. This breathing pattern is continually practiced throughout the BW session.

The one hour session is composed of five steps. During the first 10 min, the participant began with a series of specific movements to warm up both arm and leg muscles and to relax the entire body, complete deep breathing as described above, in a synchronized pattern with body movements and was mentally focus on breathing. The following 5 min were dedicated to mentally scan the body posture while walking consciously and breathing patterns were continually synchronized. In the next 25 min, the participant was engaged in a quicker walking rhythm in which inhaling and exhaling patterns are performed at different intervals and combined with silently short-repeated phrases. The walking episode was concluded in the next 5 min by gradually reducing the walking pace. In the final remaining time, a new series of resistance exercises were initiated together with stretching and concluded with an episode of meditative visualization. The physical activity of BW was imparted by a qualified doctor trained in this program. Patients practiced a 1 h session of BW, three times a week for 6 mo.

Statistical analysis

Data and statistical analysis only of the 17 patients that remained until the end of the program were included. Numeric data was grouped by mean ± SD and minimum and maximum as indicated. Results at 3 and 6 mo of the program were compared against the basal using the non-parametric Wilcoxon rank test. A P value less than < 0.05 was considered statistically significant.

RESULTS

Demographic and clinical characteristics of HCV patients that participated in the BW program are shown in Table 1. A higher proportions of women than men is in agreement with the frequency of HCV in the west of Mexico as reported before[30,31]. Normal weight was present only in 29.4% of the patients, overweight in 17.6% and obesity in 53%. Five patients had cirrhosis and the rest had fibrosis and liver steatosis. Waist circumference was 84.9 ± 10.0 cm in women and 97.6 ± 11.6 cm in men. Waist circumference above 80 cm for women and more than 90 cm for men is considered a risk factor for the development of type 2 diabetes mellitus and/or insulin resistance in Mexico[24].

Type 2 diabetes was present in 18% of the patients studied, whereas dyslipidemia was in 41.1%. Two patients had isolated hypercholesterolemia and 5 presented with isolated hypertriglyceridemia. Insulin resistance as determined by the HOMA index was present in 77% of the participants.

Anthropometric measurement in HCV patients at baseline, 3rd and 6th mo of the program are shown in Table 2. In spite of minimal changes in body weight, a reduction in BMI and waist circumference was observed during the program period, as well as a statistically significant increase in the fitness score ($P < 0.001$) at the 3rd and 6th mo.

An improvement in the lipid profile and liver function tests was observed during the BW program (Table 3). A statistically significant reduction in triglycerides was detected at 6th mo ($P < 0.01$). This reduction was

Table 2 Anthropometric measurements in chronic HCV patients at start, 3 and 6 mo of breathwalking (BW) intervention

Parameter	Unit of parameter	Basal	3 mo	6 mo
Weight	kg	69.1 ± 11.0	68.2 ± 10.8	68.4 ± 10.4
BMI	kg/m^2	27.6 ± 3.3	26.7 ± 3.6	26.5 ± 3.0[a]
Fat	%	32.3 ± 7.1	31.6 ± 7.1[b]	31.5 ± 6.6[a]
Muscle	kg	44.2 ± 7.9	45.3 ± 9.4	45.2 ± 9.4
WC	cm	88.0 ± 10.4	87.2 ± 10.0	86.0 ± 9.4[b]
Fitness	Score	76.0 ± 13.4	81.0 ± 3.0[b]	83.0 ± 2.6[d]

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ between baseline and 3 or 6 mo.

Table 3 Biochemical profile in chronic HCV patients at start, 3 and 6 mo of breathwalking (BW) (mean ± SD)

Biochemical parameter	Basal	3 mo	6 mo
Glucose (mg/dL)	94.0 ± 16.5 (77-128)	93.5 ± 21.8 (71-142)	87.2 ± 12.3 (75-121)
ALT (U/L)	106.6 ± 93.1 (18-349)	82.8 ± 63.4 (20-241)	59.4 ± 32.7[b] (17-112)
AST (U/L)	101.4 ± 66.6 (32-265)	87.8 ± 61.8 (20-226)	84.7 ± 61.8 (24-241)
ALT/AST ratio	1.04	0.94	0.70[b]
Total bilirubin (mg/dL)	1.38 ± 1.2 (0.30-5.2)	1.22 ± 1.1 (0.29-4.9)	0.62 ± 0.36[b] (0.0-1.1)
Cholesterol (mg/dL)	157.1 ± 86.5 (103-231)	148.0 ± 38.4 (82-217)	146.0 ± 39.8 (67-205)
Triglycerides (mg/dL)	165.0 ± 86.5 (86-378)	141.9 ± 46.4 (85-210)	124.6 ± 49.2[b] (64-197)
TG/HDL ratio	4.0	3.9	2.7[a]

Data are expressed in mean ± SD. TG/HDL: Triglycerides/high density lipoprotein. [a]$P < 0.05$, [b]$P < 0.01$ between baseline and 3 or 6 mo.

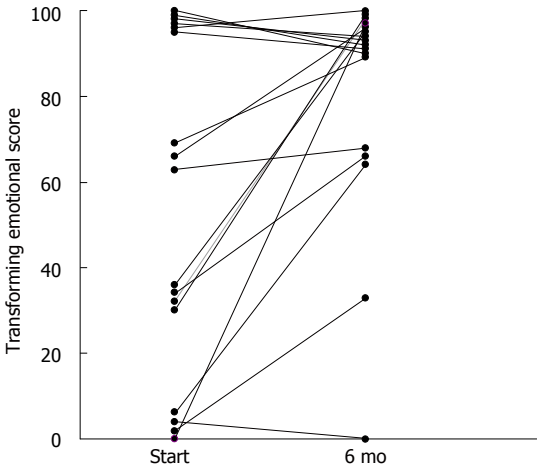

Figure 1 Patients' mood state with respect to disease at the beginning (start) and at the end of breathwalk (BW) after 6 mo. Wilcoxon rank test. $P < 0.01$ between start and 6 mo.

associated with a statistically significant decrease of the TG/HDL ratio. Improvement in liver function tests was documented by a statistically significant decrease in bilirubin ($P < 0.01$) and ALT serum levels after six months ($P < 0.01$), as well as in the ratio ALT/AST ($P < 0.01$).

Viral load was documented only in 7 patients at the beginning and at the end of the BW program. A reduction of the viral load was detected in 4 patients; from 78667 ± 50000 copies baseline levels to 600 ± 0 at the end of BW intervention. Patients that showed an improvement in the viral load had HCV genotype 1b, 2a, 2c and 3. In the other three patients no changes in viral load were detected.

At the beginning of the program, 47% of the HCV patients expressed negative mood states (depression, anger, anguish, fear). At mo 6 of BW, 94% of the patients presented with positive mood states (Figure 1). At the end of BW, the patients report improved emotional state ($P < 0.001$) compared with the beginning in all categories (Table 4). In general, the majority of the patients were motivated towards physical activity, optimistic towards life, and they referred being happy.

Table 4 Mood state based on transformed score evaluation at start, 3 and 6 mo of breathwalking (BW)

Mood state category	Transformed score	Baseline *n* (%)	3 mo *n* (%)[b]	6 mo *n* (%)[b]
Excellent	100	6 (35)	11 (65)	15 (88)
Good	66.66	3 (18)	5 (29)	1 (6)
Regular	33.33	4 (23.5)	0 (0)	1 (6)
Bad	0	4 (23.5)	1 (6)	0 (0)

Wilcoxon rank test; [b]$P < 0.001$ between start and 3 or 6 mo.

DISCUSSION

Obesity and DM2 are an epidemiological problem worldwide, associated with changes in lifestyle. Traditional homeland dietary and physical activity habits have shifted to a mixture of occidentalized and traditional diets combined with more sedentary life. Therefore, the extent of obesity and DM2 in HCV infected patients could be expected to vary in each continent or country mainly due to the diversity of race and environmental factors present in the population. More than fifty percent of the adults are overweight or obese in Mexico[32], and the association of HCV infection with obesity, which by itself is associated with insulin resistance[33], feasibly explains why 77% of the studied patients presented with IR at the time of the study. Hypercholesterolemia and hypertriglyceridemia were the two main dyslipidemia present in 41% of the participants. Indeed, hypertriglyceridemia has been associated with insulin resistance and development of DM2[34]. Therefore, a further rise in DM2 incidence could be expected in these HCV-infected patients in a near future. This reinforces the need to establish specific strategies based on lifestyle changes in order to prevent further metabolic abnormalities or deterioration of liver function.

In order to analyze the effect of BW on lipid profile, only patients that did not vary their dietetic habits during the program were evaluated. A statistically significant decrease in triglycerides at mo 6 of the program was observed as well as the decrease of the TG/HDL ratio. Therefore, BW had an important effect on both types of dyslipidemia and IR without a dietary intervention as seen in other studies[16,17,35]. However, the inclusion of an individualized dietetic program considering their own culture and specific habits could exert a further decrease on dyslipidemia and BMI.

The BW intervention achieved an important effect on the fitness score with a statistically significant increase at 3 mo of the program. The fitness score is an impedance measurement that reflects the muscle/fat ratio, which explains the lack of significant changes in weight, especially during the initial weeks or month of the exercise.

It has been shown that walking requires a longer time period to exert its beneficial effect on lipid metabolism in obese and patients with DM2[36,37]. An advantage that BW has over conventional walking is that it is an aerobic exercise where the patients maintain up to 70% VO_{2max} through rhythmic walking, and during the same session resistance exercise is included and associated with mental attention. Furthermore, it has been shown that anaerobic exercise (exercise load), which could be harmful for the patient with chronic liver disease, does not have significant effect on lipid metabolism at shorter periods of time[38].

An important effect of BW on liver function was also observed in HCV-infected patients. A statistically significant decrease of ALT, the ratio ALT/AST and total bilirubin was detected at mo 6 of the program as well as a decrease of the viral load in four of seven patients, where RNA-HCV was quantified at the beginning and at the end of the program. Little is known about the physiopathological mechanism related to the effect of exercise on the improvement of liver function tests in patients with chronic liver disease. However, even when it is known that exercise can improve insulin resistance at the cellular level[39], it can be speculated that a decrease in fatty liver would cause less liver injury, although further studies are required in order to determine any proposed

mechanism. Also, the small number of analyzed patients with changes in viral load may only indicate the importance of performing further studies with this approach, primarily in those patients that have not responded to antiviral treatment[14] either because of the virus genotype[40] or as a secondary effect of the interferon by itself[41].

At the beginning of the program, only 12% of the patients reported a positive mood state, however, negative mood state shifted towards a positive mood score in 88% of the patients at the end. Most studies focus on analyzing the effect of exercise on metabolic parameters, yet it is unusual to evaluate the emotional state of the patients[42]. The observed significant change in mood state and therefore in quality of life may be attributed to the synchronized breathing patterns combined with walking throughout the BW routine. A meditative state of mind is provoked that allows disconnection from the external environment and liberates negative thought and feeling[43]. Another important factor that may have contributed to the results in this study is the fact that, throughout the six-month program, a close relationship was established between the instructor and the patients. This could have enhanced compliance and adherence of the patients to exercise and therefore to the changes in habits.

The fact that in the past and even in current days exercise has been controindicated in patients with chronic liver disease appears to be proven wrong, since in this study, chronic HCV patients with fibrosis and compensated cirrhosis improved in their metabolic and hepatic profiles at the end of the program. This approach is important because it can be incorporated as a complementary treatment in patients who are candidates for interferon treatment including non-responder patients. However, further studies should be continued to evaluate the effect of an integrative management of the patient that includes antiviral treatment, specific types of exercise, diet and mood state.

In conclusion, this study identifies a specific effect of BW on anthropometric, metabolic and liver functional tests, and demonstrates the importance of the patient's mood state to enhance compliance to the program. Furthermore, this approach could be used in patients living in developing countries, where the cost of the antiviral treatment is expensive and in the mean time government authorities and/or international institutions can aid with financial funding to treat these patients.

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COMMENTS

Background

In the last few years, two different issues have been on top of the table in the hepatology field: the association of obesity and type 2 diabetes in patients infected with hepatitis C virus versus the traditional medical indication of resting in patients with chronic liver disease and, on the other hand, the current problem of the nonresponder patient to antiviral treatments, especially in those who are overweight or obese.

Research frontiers

Whereas it is clear that an increase in physical activity and/or specific exercises are indicated in overweight, obese and type 2 diabetes patients, there are few studies related to the effect of exercise on metabolic and hepatic function parameters in patients with liver disease and even to further approach patient's emotions and well-being. Such strategy intends to involve different research areas so to achieve an integrative evaluation of patients that includes the study of metabolic parameters, liver function tests, emotional state, diet and exercise that are otherwise accessed in an individual manner.

Innovations and breakthroughs

This study represents an initial attempt to use Breathwalk as a specific technique in chronic liver disease patients, and to test in the near future the effect of different physical activity and exercise strategies on metabolic, liver function and anthropometric parameters, as well as emotional states in these patients.

Applications

Breathwalk is an innovative exercise technique that is easy to perform which could be implemented as a tool for patients with chronic liver diseases, especially at early stages of disease and in other chronic pathological conditions such as obesity, metabolic syndrome and type 2 diabetes.

Terminology

Breathwalk: An exercise technique that synchronizes walking steps, specific breathing patterns and focused attention. No-responders: Patients who do not achieve to lower viral loads after administration of the standard doses of antiviral monotherapy or combination therapy after (months) treatment.

Peer review

The manuscript studies the impact of breathwalking exercise on metabolic and fitness paramters in patients with chronic hepatitis C infection. It is interesting and of possible relevance to the field.

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RAPID COMMUNICATION

Inflammatory cytokines suppress arylamine *N*-acetyltransferase 1 in cholangiocarcinoma cells

Benjaporn Buranrat, Auemduan Prawan, Banchob Sripa, Veerapol Kukongviriyapan

Benjaporn Buranrat, Auemduan Prawan, Veerapol Kukongviriyapan, Department of Pharmacology, Faculty of Medicine, Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen 40002, Thailand
Banchob Sripa, Department of Pathology, Faculty of Medicine, Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen 40002, Thailand
Supported by Khon Kaen University Research Fund, Grant from National Science and Technology Development Agency through the Research-Team-Strenghtening Grant Scheme 2006
Correspondence to: Veerapol Kukongviriyapan, Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. veerapol@kku.ac.th
Telephone: +66-43-348397 Fax: +66-43-348397
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Abstract

AIM: To evaluate the effect of inflammatory cytokines on arylamine *N*-acetyltransferase 1 (NAT1), which is a phase-Ⅱ enzyme involved in the biotransformation of aromatic and heterocyclic amines found in food, drugs and the environment.

METHODS: Human cholangiocarcinoma KKU-100 cells were treated with a mixture of proinflammatory cytokines (interferon-γ, interleukin-1β, and tumor necrosis factor-α) for 48 h, and the effect on NAT1 activity was assessed by high performance liquid chromatography, while *NAT1* expression was determined by reverse-transcription polymerase chain reaction. The oxidative stress on the cells was examined by the formation of nitric oxide, superoxide anion and glutathione (GSH) levels. The cells were also treated with *S*-nitroso-glutathione (GSNO), a nitric oxide donor, to see if the responses were similar to those obtained with the inflammatory cytokines.

RESULTS: Cytokines suppressed NAT1 activity, reducing the V_{max} without affecting the K_m. Cytokines also had a significant impact on the induction of nitric oxide production and in reducing the redox ratios of glutathione (GSH) and GSH disulfide. Treatment with GSNO for 2-48 h reduced NAT1 activity without affecting the GSH ratio. Moreover, inflammatory cytokines and GSNO suppressed NAT1 mRNA expression.

CONCLUSION: These findings indicate an association between inflammation and suppression of NAT1, which perhaps contributes to chemical-mediated toxicity and carcinogenesis.

Key words: Arylamine *N*-acetyltransferase 1; Phase Ⅱ drug-metabolizing enzyme; Inflammatory cytokine; Oxidative stress; Cholangiocarcinoma

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INTRODUCTION

Arylamine *N*-acetyltransferases (NATs) are well known polymorphic phase-Ⅱ drug-metabolizing enzymes. Human NAT1 and NAT2 are encoded by two closely related *NAT1* and *NAT2* genes[1,2]. NAT1 mRNA and protein are expressed in a wide range of tissues, whereas NAT2 mRNA and protein are present mainly in the liver and the gastrointestinal tract[3]. Biliary epithelial cells do not express NAT2 but retain NAT1 activity[4].

NATs are important enzymes capable of acetylation reactions, which are involved in the detoxification and metabolic activation of various chemicals in drugs, food and the environment[5]. *N*-acetylation is usually considered a detoxification process, because it renders the nitrogen atom less susceptible to oxidation, a process primarily mediated by cytochrome P450 1A2[6]. In contrast, *O*-acetylation is an activation step for heterocyclic and aromatic amines.

Genetic polymorphisms of NAT1 have been identified, but the relationship between genotypes and phenotypes is not clear[7]. Several studies have suggested that NAT1 and NAT2 acetylation polymorphism plays a role in carcinogenesis in humans exposed to certain carcinogenic chemicals[5]. Our previous studies suggested that *NAT1* and *NAT2* polymorphisms may be modifiers of individual risk for cholangiocarcinoma, a cancer of the biliary epithelium[8]. Overexpression of NAT1 in breast tumors is associated with growth properties, as well as chemotherapeutic drug resistance[9] and drug allergy, in particular, cutaneous drug reactions associated with sulfonamides, whereas inactivation of the enzyme may contribute to drug toxicity and cancer risk[10,11]. More recently, NAT1 activity was shown to

be suppressed by oxidant species such as hydrogen peroxide (H_2O_2) and peroxynitrite[12,13]. Peroxynitrite and H_2O_2 irreversibly inactivate NAT1 by oxidation of its conserved catalytic cysteine residue to sulfinic or sulfonic forms[12,13].

Chronic infection and inflammation are important risks factors for several cancers, including cholangiocarcinoma, a highly malignant adenocarcinoma originating from the cholangiocytes. The highest incidence of cholangiocarcinoma worldwide is seen in Northeast Thailand[14]. Infection of the biliary system with liver fluke (*Opisthorchis viverrini*) and possibly exposure to carcinogenic chemicals are believed to be causally related to cholangiocarcinoma[15,16]. The association of cholangiocarcinoma and liver fluke was observed in hospital case series which showed an excessively increased risk in patients with liver fluke infestation. Inflammation of the biliary tract caused by mechanical injury and the release of metabolic products from the flukes, together with the damaging effects of reactive metabolites from endogenous and environmental chemicals have been proposed as the responsible factors[17], which induce alterations in gene expression resulting to cellular hyperproliferation and development of neoplasia[15,16]. In an animal model of cholangiocarcinogenesis, hamster livers infected with liver flukes showed inflammation of the bile duct epithelium, and contained 8-oxo-deoxyguanosine and 8-nitroguanine adducts, which are biomarkers of DNA attack by reactive oxygen and nitrogen species[18]. Thus, inflammatory processes can cause oxidative stress and thereby affect NAT1 activity.

In the present study, we examined the effect of a combination of proinflammatory cytokines on KKU-100 cholangiocarcinoma cells. We also assessed the effect of cytokines on NAT1 activity and the development of oxidative stress; some of these effects were reproduced by a nitric oxide donor.

MATERIALS AND METHODS

Human CCA cell line

The human biliary epithelial cell line KKU-100, derived from intrahepatic cholangiocarcinoma, was established in our institute[19]. The cells were cultured in Ham's F12 containing 4 mmol/L L-glutamine, 1 mmol/L Na-pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin and 10% fetal bovine serum and maintained under an atmosphere of 5% CO_2 at 37℃. The media was renewed every 3 d. The cells were trypsinized with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) and subcultured in the same media. Twenty four hours after subculture, cells at approximately 70% confluence were exposed to a combination of inflammatory cytokines consisting of human interleikin-1β (IL-1β) (1 ng/mL), interferon-γ (IFN-γ) (400 U/mL), and tumor necrosis factor-α (TNF-α) (500 U/mL) (Biosource International Camarillo, CA) for 48 h or S-nitroso glutathione (GSNO, 100 μmol/L) for 2 or 48 h. In experiments determining nitrite production, KKU-100 cells were cultured in non-phenol red medium.

Biochemical assays

Nitrite assay: After treatment of the cell cultures, the accumulation of nitrite in the culture medium was assessed by mixing an equal volume of the medium with Griess reagent (containing 0.1% N-1-napthylethylenediamine in water and 1% sulfanilamide in 5% H_3PO_4). Absorbance was read at 540 nm with an ELISA plate reader.

Superoxide production: KKU-100 cells were cultured in 35-mm dishes with cytokines for 48 h. Cell cultures were washed with Tris-buffered saline (TBS) (10 mmol/L Tris HCl and 150 mmol/L NaCl, pH 7.3) and incubated for 30 min with phorbol-12-myristate-13-acetate (PMA) (0.68 μg/mL) or with N^G-nitro-L-arginine methylester (L-NAME) (100 μmol/L) or for 5 min with NADPH (200 μmol/L). Lucigenin (100 μmol/L) was added to the culture dishes, and chemiluminescence was recorded using a luminometer (Luminometer model 20/20^n, Turner Biosystems, CA).

Assay of GSH and glutathione disulfide: After treatment with cytokines, the cells were trypsinized and washed with cold tris buffer saline (TBS) by centrifugation at 1500 × *g* at 4℃ for 10 min and resuspended in TBS buffer. One hundred microliters of cell suspensions were reacted with 10 μL of 1-methyl-2 vinylpyridinium triflate (M2VP) (3.3 mmol/L) as a GSH scavenger for assay of GSH disulfide (GSSG)[20] or with distilled water for assay of total GSH, and cell suspensions were stored frozen at -20℃ until analysis. Total GSH and GSSG were assayed according to the Tietze method[21]. The amount of reduced GSH was calculated from total GSH and GSSG. Another aliquot of cell suspensions was used to determine protein content in a Bradford dye binding assay with bovine serum albumin as the standard.

Assay of NAT1 activity: NAT1 enzyme activity was assayed using high performance liquid chromatography according to a previously described method[4], with some modifications. Briefly, the cell cultures were washed with TBS and scraped into a microcentrifuge tube with lysis buffer (1X cell lysis buffer containing 1 mmol/L dithiothreitol [DTT] and 0.1 mmol/L phenylmethylsulfonyl fluoride [PMSF]). The cells were vortexed and centrifuged at 12000 × *g* at 4℃ for 30 min. The supernatant (cytosol) was stored in 10% (v/v) glycerol. The cytosol protein was used for assays of protein concentration and NAT1 activity. The reaction mixture consisted of 40 μL cytosol (final concentration 50 μg/mL), 20 μL acetyl CoA-regenerating system (DL-acetylcarnitine, 5.4 mg/mL, carnitine acetyltransferase (1 U/mL) in NAT assay buffer (225 mmol/L triethanolamine HCl, 4.5 mmol/L EDTA, and 4.5 mmol/L DTT, pH 7.5), and 20 μL acetylCoA (final concentration 100 μmol/L). The reaction was initiated by addition of 10 μL *p*-aminobenzoic acid (PABA) in 2.5% dimethylsulfoxide (final concentration 1.25-100 μmol/L) and incubated for 30 min. The reaction was stopped by addition of 10 μL of 15% perchloric acid and centrifuged at 12000 × *g* at 4℃ for 10 min. The supernatant was injected directly onto a high performance liquid chromatography column (YMC-PACK Pro-C_{18}, 5 μm, 150 mm × 4.6 mm; YMC Co., Japan) and

A

Treatment	K_m (μmol/L)	V_{max} (pmol/min per mg protein)
Control	2.8 ± 0.3	211.2 ± 2.9
Cytokine mixture	3.3 ± 0.2	135.7 ± 3.3[a]

Values are shown as mean ± SE.

B

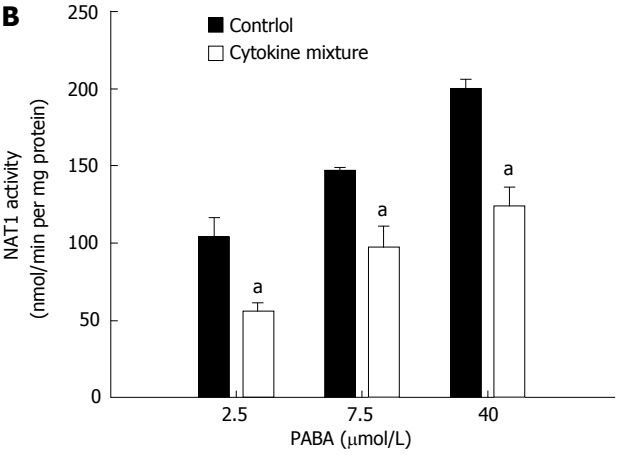

Figure 1 The effect of a mixture of inflammatory cytokines on NAT1 activity in KKU-100 cells. Assays were performed with the cytosol fraction extracted from KKU-100 cells and treated with a combination of cytokines for 48 h. **A**: Kinetic analysis of NAT1 activity; **B**: Activity of NAT1 at various substrate concentrations (PABA) of KKU-100 cells with or without treatment with cytokines. [a]$P < 0.05$ between cytokine treated and control groups.

eluted with a mobile phase consisting of water:acetonitrile: acetic acid:triethylamine:tetrahydrofuran (90.2:8.5:1:0.05:0.25 v/v) at a flow rate of 1.0 mL/min. *N*-acetyl-aminobenzoic acid (Ac-PABA) was detected using a fluorescence detector (Waters 740 scanning fluorescence detector; Waters Corp, Milford) set for excitation at 270 nm and emission at 340 nm. Analytical precision was evaluated by intra- and inter-day assay validation. The coefficients of variation were less than 5% and 10%, respectively. The detection limit of Ac-PABA was less than 1 pmol.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted from KKU-100 cells using Trizol®LS reagent according to the manufacturer's instructions. Total RNA (3 μg) was reverse-transcribed in a 20 μL volume containing 0.5 μg oligo(dT)$_{15}$ primer, 20 units RNasin® ribonuclease inhibitor and ImProm-Ⅱ™ reverse trancriptase (Promega, Madison, WI) in 10x polymerase chain reaction (PCR) buffer, 3 mmol/L $MgCl_2$, and 1 mmol/L dNTPs. The first-strand cDNA was synthesized at 42℃ for 60 min. Reverse transcription products were used as a template for PCR. PCR amplification was performed using specific primers for *NAT1* and farnesyl-diphosphate farnesyltransferase1 *(FDFT1)* as an internal control. The PCR primer sequences were: *NAT1* forward primers, 5'-CCTAGAAGACAGCAAATACCG-3'; *NAT1* reverse primers, 5'-AGCCCACCAAACAGTGA-3' (PCR product: 170 bp); *FDFT1* forward primers, 5' -TTTAACTTC TGTGCTATTCCAC-3'; *FDFT1* reverse primers, 5'-TCTCCAGTCTGAACATAGTC-3' (size of PCR product: 325 bp).

PCR was performed in a final volume of 25 μL containing cDNA template, 1.5 μmol/L of each *NAT1* primer or 0.4 μmol/L of each *FDFT1* primer, 1 U Platinum® Tag DNA polymerase (Invitrogen, Carlsbad, CA), 3 mmol/L $MgCl_2$, and 0.8 mmol/L dNTPs using a Px2 Thermal Cycle (Thermo Electron, Milford, MA). After an initial denaturating step at 94℃ for 5 min, 32 PCR cycles were performed for NAT1 and 28 cycles for FDFT1, as follows: denaturating for 1 min at 94℃, annealing for 1 min at 55℃, and extension for 1 min at 72℃. The final extension was performed at 72℃ for 10 min. The PCR products were separated by electrophoresis on a 3% agarose gel containing ethidium bromide. Gels were visualized and photographed. Band density was analyzed with Gel-Pro3 software. The relative amount of NAT1 mRNA was expressed as a ratio of FDFT1 mRNA.

Statistical analysis

Data are expressed as mean ± SE of duplicate assays from three independent experiments. Student's *t*-test was used to determine significant differences between each experimental group. The level of significance was set at $P < 0.05$.

RESULTS

Effect of the cytokine mixture on the kinetics of NAT1 acetylation

NAT1 activity of KKU-100 cells and Michaelis-Menten constants for PABA *N*-acetylation are shown in Figure 1A. Treatment with the mixture of inflammatory cytokines for 48 h did not affect cell viability (data not shown) but resulted in a significant decrease in $V_{max(apparent)}$ without affecting $K_{m(apparent)}$ (Figure 1A). This implies a change in the amount of enzyme but not in its affinity. The initial velocities of PABA *N*-acetylation by the cytosolic enzyme from KKU-100 cells are shown in Figure 1B.

Effect of the cytokine mixture on production of nitric oxide and superoxide

Since direct application of oxidant species has been reported recently to suppress NAT1 activity, our experiment showed that treatment with a mixture of cytokines could induce oxidative stress by overproduction of nitric oxide, detected by nitrite assay. Whereas basal production of nitric oxide by KKU-100 cells was nearly absent, production was greatly stimulated by inflammatory cytokines (Figure 2A).

To determine if KKU-100 cells were capable of releasing superoxide anion, aggravating oxidative stress, the cells were cultured with a mixture of cytokines. The cells exhibited low basal release of superoxide; the levels increased slightly after cytokine treatment (Figure 2B). To determine whether the low levels of superoxide were due to scavenging subsequent to overproduction of nitric oxide, the cells were treated with a nitric oxide synthase inhibitor. Inhibition of nitric oxide synthases by L-NAME did not increase superoxide levels relative to the control or baseline values (Figure 2B). When the cells were incubated with NADPH, the substrate of NADPH oxidases, superoxide production in the control and cytokine-treated groups was greatly increased and reached similar levels. Furthermore, treatment with PMA, a protein kinase C activator, resulted in a marked increase in superoxide production in the cytokine-treated group but not in the controls (Figure 2B).

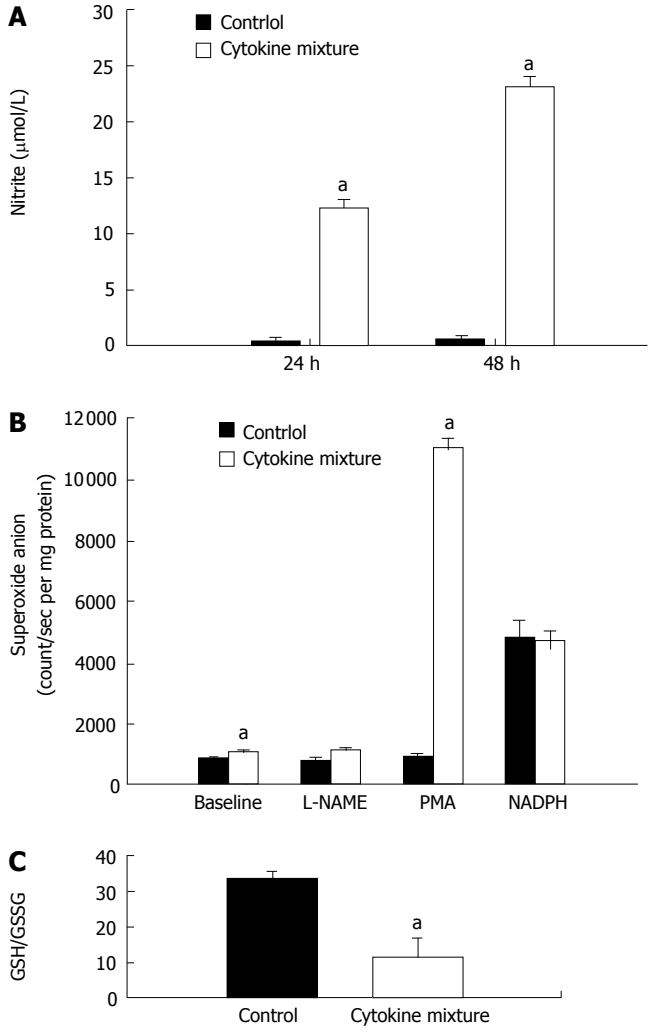

Figure 2 Oxidant status of KKU-100 cells after exposure to a mixture of inflammatory cytokines. **A**: Stimulation of nitric oxide production, assayed as nitrite levels. Cultured media was collected at 24 and 48 h after exposure; **B**: Superoxide formation. Cell cultures were washed and incubated with PMA (0.68 μg/mL), L-NAME (100 μmol/L), or NADPH (200 μmol/L), and superoxide production was measured using the chemiluminescence method; **C**: Redox status was assessed as the GSH and GSSG ratio. Results are presented as mean ± SE from 3 separate experiments. $^{a}P < 0.05$ between cytokine treated and the respective control groups.

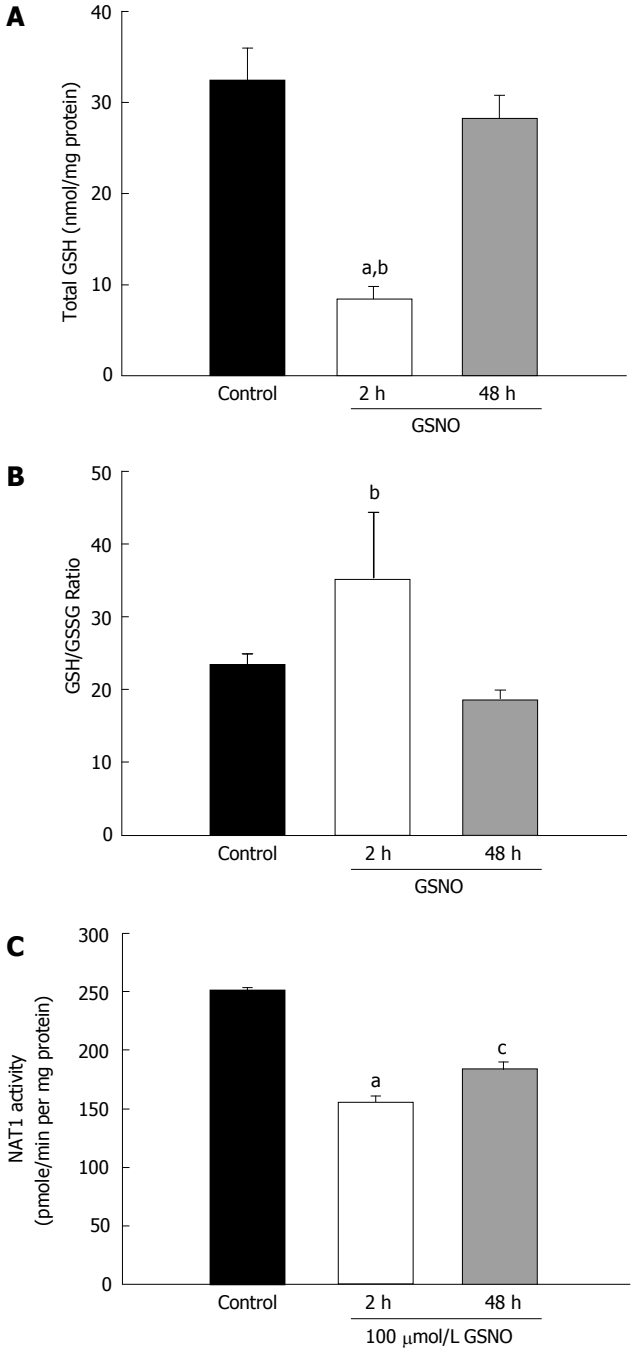

Figure 3 Effect of treatment with the nitric oxide donor GSNO on KKU-100 cells. Cell cultures were treated with 100 μmol/L GSNO for 2 and 48 h. **A**: Total GSH in the cells; **B**: GSH/GSSG ratio; **C**: Activity of NAT1 PABA-acetylation. Bars represent the mean ± SEM from 3 separate experiments. $^{a}P < 0.05$ *vs* control groups, $^{c}P < 0.05$ *vs* the 48-h treatment group.

Effect of the cytokine mixture on GSH levels

Since treatment with inflammatory cytokines may result in the formation of free radicals, further experiments were performed to determine if treatment will alter GSH levels and the oxidative status. KKU-100 cells exposed to the cytokine combination for 48 h showed no significant change in the total GSH levels (control: 36.7 ± 2.8 nmol/mg protein, cytokine-treated: 31.9 ± 14.5 nmol/mg protein). However, there was a marked reduction in redox status (GSH/GSSG ratio; Figure 2C) of the treated cells.

Effect of GSNO on NAT1 activity and redox status

The above noted experiments demonstrated that the mixture of cytokines induced nitric oxide production and oxidative stress. The next experiment investigated whether a nitric oxide donor would produce similar results. Treatment with GSNO elicited a very large decrease in total GSH within 2 h; however, the GSH levels normalized to the control levels at 48 h (Figure 3A). There was no significant change in the GSH/GSSG ratio after GSNO treatment (Figure 3B). However, treatment with GSNO reduced NAT1 activity as early as 2 h, and the suppression persisted at 48 h (Figure 3C).

Effect of the cytokine mixture and GSNO on expression of NAT1

Reverse transcription PCR was used to assess the effects of proinflammatory cytokines and nitric oxide donors on the expression of NAT1 mRNA. The results are shown in

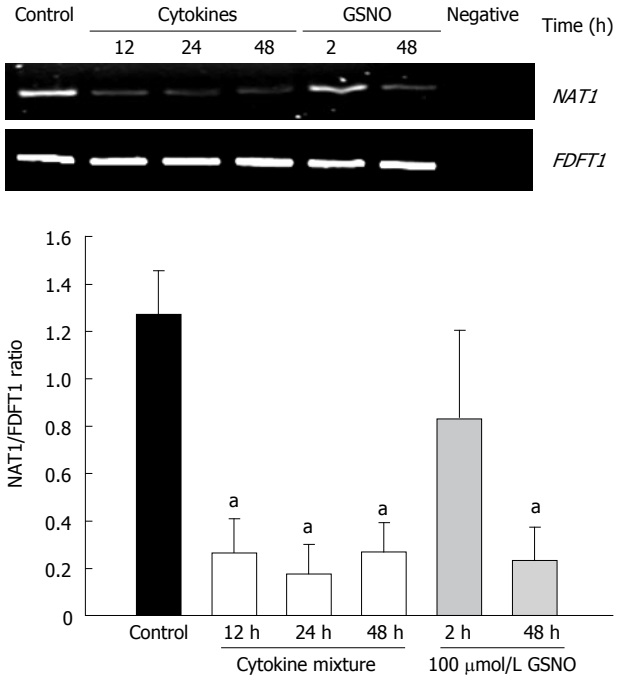

Figure 4 Effect of the cytokine mixture and a nitric oxide donor on the expression of NAT1 in KKU-100 cells. KKU100 cells were incubated with a mixture of cytokines for 48 h or with 100 μmol/L GSNO for 2-48 h. Cells were harvested, and RNA was extracted and analyzed by reverse transcription PCR using FDFT1 as an internal control. $^{a}P < 0.05$ *vs* controls.

Figure 4. Cytokine treatment elicited a decrease in NAT1 mRNA levels. Similarly, GSNO resulted in reduction of the NAT1 mRNA at 48 h, but not at 2 h.

DISCUSSION

The catalytic activity of NAT enzymes is dependent on a reactive cysteine residue, since its activity is inhibited when this residue is modified by *N*-hydroxy-arylamine compounds, hydroxamic acids[22], enzyme substrates[10], as well as oxidant species[12,13,23]. Oxidative stress and chronic inflammation are inseparable such that inflammation inevitably produces oxidant species, resulting in tissue damage[24]. The present study shows that proinflammatory cytokines suppress NAT1 activity similar to treatment with oxidant species[12,13]. Kinetic analysis of NAT1 showed a significant decrease in V_{max}, suggesting that the reduction of NAT1 activity was due to a decrease in the enzyme level. This alteration was probably due to enzyme inactivation or suppression of *NAT1* expression.

Inflammatory cytokines induce the expression of inducible nitric oxide synthase (iNOS) and increase nitric oxide production in cholangiocytes[25]. Our results are consistent with this finding, in fact the basal nitric oxide production was very low but the production increased markedly after treatment with inflammatory cytokines. It is plausible that induction of nitric oxide formation is partly responsible for the suppression of NAT1 activity. Treatment with a nitric oxide donor inhibited NAT1 in a manner similar to cytokine treatment, supporting the possibility that nitric oxide and perhaps peroxynitrite play an important role in modulation of NAT1 activity.

A previous study showed that treatment of breast cancer cells with peroxynitrite irreversibly inactivated NAT1[12]. Peroxynitrite is formed by a reaction between nitric oxide and superoxide anion. In the present study, inflammatory cytokines did not induce a large increase in superoxide, even when nitric oxide formation was inhibited by L-NAME. However, NADPH oxidases, which are membrane bound enzymes that require assembly of subunits from cytosol to become fully functional, are responsible for the formation of superoxide in phagocytic and nonphagocytic cells[26]. They may be upregulated by inflammatory cytokines, as has been shown in smooth muscles and kidney cells[27]. The NADPH oxidases in KKU-100 cells may also be upregulated, however stimulation by PMA, a protein kinase C activator that mediates phosphorylation and recruitment of oxidase subunits[26] may be required to render the NAT1 enzyme fully functional. Together, these findings suggest that superoxide and perhaps peroxynitrite did not play a major role in the cytokine-induced suppression of NAT1 activity in KKU-100 cells. Nevertheless, the role of superoxide and peroxynitrite *in vivo*, a situation where infiltrating macrophages are present, has not been investigated.

The ability of inflammatory cytokines to induce oxidative stress was clearly evident in the present study, as there was a marked increase in pro-oxidant status, evidenced by a decrease in the redox ratio of GSH/GSSG in cultured cells exposed to the mixture of cytokines. In contrast, treatment with GSNO did not affect the redox ratio at any time point. However, total GSH, representing the major intracellular antioxidant pool, decreased substantially after GSNO treatment, especially at the 2-h time point. Altogether, the differences in the effects of cytokines and GSNO suggest that the effects of the cytokine mixture were mediated by nitric oxide and redox-sensitive pathways.

A recent report has shown that intrahepatic cholangiocarcinoma seen in liver fluke endemic areas is characterized by altered expression of drug metabolizing genes, whereas that from non-endemic areas such as Japan shows alteration in the growth factor signaling genes[17]. This may indicate that drug metabolizing genes are involved in the metabolism of potential carcinogenic chemicals. However, information regarding NAT1 expression is currently not available. Regulation of *NAT1* expression has been under study for a long time[23,28]. Previous studies have shown that suppression of NAT1 activity by substrates or oxidant species is due to direct inhibition of the enzyme molecules[13,29], since the expression of NAT1 mRNA does not show any alteration[29]. Our study is the first to demonstrate that treatment with a mixture of cytokines suppresses NAT1 mRNA expression. Using deletion mutant constructs, a promoter site for basal *NAT1* expression was identified[30] comprising of an activator protein 1 (AP-1) binding site. Transcription factor AP-1 is upregulated or downregulated by oxidative stress and inflammatory cytokines, depending upon the oxidant levels and the type of inflammatory cytokines[31,32]. Suppression of *NAT1* expression in KKU-100 cells may involve down regulation or inactivation of AP-1, although specific evidence in this regard remains

to be established. Direct inhibition of NAT1 by oxidant species (nitric oxide) cannot be ruled out, particularly in *in vivo* conditions; this may occur concurrently with suppression of expression.

In summary, treatment with inflammatory cytokines suppresses NAT1 activity and mRNA expression in cholangiocarcinoma KKU-100 cells. This suppression was associated with oxidative stress and nitric oxide production. These findings show that inflammation can suppress NAT1, a key cellular defense enzyme. Moreover, such a suppression may be implicated in drug toxicity and cancer risk.

ACKNOWLEDGMENTS

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COMMENTS

Background

Arylamine *N*-acetyltransferases-1 (NAT1) is an important phase Ⅱ drug metabolizing enzyme that is constitutively expressed in most tissues. Its activity is inactivated by oxidant species.

Research frontiers

NAT1 expression has been demonstrated in cholangiocarcinoma cells (CCA), and polymorphism of NAT genes has been implicated as a risk factor for cancer. Inflammation of the bile duct resulting from opisthorchiasis may alter the activity of the drug metabolizing enzymes including cytochrome P450 and NAT1. Modulation of NAT1 activity may be implicated in drug induced-toxicity and carcinogenesis.

Innovations and breakthroughs

Suppression of NAT1 activity and gene expression is associated with cytokine-induced oxidative stress. A combination of proinflammatory cytokines induces changes in cellular redox and nitric oxide production in CCA and these may be involved in down-regulation of NAT1.

Applications

NAT1 activity could be modulated by inflammation and this may be associated with drug induced toxicity and carcinogenesis.

Terminology

Opischorchiasis: liver fluke (Opisthorchis viverrini) infection of the bile duct.

Peer review

This manuscript describes the intriguing idea that inflammatory cytokines, which are known to be upregulated in cholangiocarcinoma, may function in part by suppressing the activity and/or expression of NAT1, an enzyme thought to be involved in the detoxification of xenobiotics. The authors also show a parallel increase in oxidative stress and nitric oxide after treatment with inflammatory cytokines. Treatment of the cell line with nitric oxide donors also shows a similar suppression of NAT1 activity and expression.

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RAPID COMMUNICATION

Predictors of premature delivery in patients with intrahepatic cholestasis of pregnancy

Jurate Kondrackiene, Ulrich Beuers, Rimantas Zalinkevicius, Horst-Dietmar Tauschel, Vladas Gintautas, Limas Kupcinskas

Jurate Kondrackiene, Limas Kupcinskas, Department of Gastroenterology, Kaunas University of Medicine, Kaunas, Lithuania
Ulrich Beuers, Department of Gastroenterology and Hepatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
Rimantas Zalinkevicius, Institute of Endocrinology, Kaunas University of Medicine, Kaunas, Lithuania
Horst-Dietmar Tauschel, Dr. Falk Pharma GmbH, Freiburg, Germany
Vladas Gintautas, Department of Obstetrics and Gynecology, Kaunas University of Medicine, Kaunas, Lithuania
Supported in part by a Grant from the Science Foundation of Kaunas University of Medicine and by Dr. Falk Pharma GmbH, Freiburg, Germany
Correspondence to: Jurate Kondrackiene, MD, PhD, Department of Gastroenterology, Kaunas University of Medicine, Eiveniu Street 2, Kaunas 50009, Lithuania. jukond@takas.lt
Telephone: +370-37-326896 Fax: +370-37-326508
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Abstract

AIM: To evaluate the predictive value of clinical symptoms and biochemical parameters for prematurity in intrahepatic cholestasis of pregnancy (ICP).

METHODS: Sixty symptomatic patients with ICP were included in this retrospective analysis. Preterm delivery was defined as delivery before 37 wk gestation. Predictors of preterm delivery were disclosed by binary multivariate logistic regression analysis.

RESULTS: Mean time of delivery was 38.1 ± 1.7 wk. No stillbirths occurred. Premature delivery was observed in eight (13.3%) patients. Total fasting serum bile acids were higher (47.8 ± 15.2 *vs* 41.0 ± 10.0 μmol/L, $P < 0.05$), and pruritus tended to start earlier (29.0 ± 3.9 *vs* 31.6 ± 3.3 wk, $P = 0.057$) in patients with premature delivery when compared to those with term delivery. Binary multivariate logistic regression analysis revealed that early onset of pruritus (OR 1.70, 95% CI 1.23-2.95, $P = 0.038$) and serum bile acid (OR 2.13, 95% CI 1.13-3.25, $P = 0.013$) were independent predictors of preterm delivery.

CONCLUSION: Early onset of pruritus and high levels of serum bile acids predict preterm delivery in ICP, and define a subgroup of patients at risk for poor neonatal outcome.

Key words: Intrahepatic cholestasis; Delivery; Pregnancy

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INTRODUCTION

Liver disorders during pregnancy range from benign nuisance to progressive and potentially lethal disorders for mothers and/or children. This is exemplified by intrahepatic cholestasis of pregnancy (ICP), which starts with modest itching and can end in intrauterine fetal demise[1]. ICP is a liver disorder unique to pregnancy and disappears after delivery. However, it frequently recurs in subsequent pregnancies or when women begin taking oral contraceptives.

The condition is very common in Chile and Bolivia (6%-27%), and in Sweden (1%-1.5%). The incidence of ICP is lower elsewhere in Europe (0.1%-1.5%) and the United States (0.7%)[2,3]. Genetic predisposition and hormonal factors have crucial roles in the pathogenesis[2]. There is increasing evidence that genetically determined dysfunction of canalicular transporters may be a risk factor for development of ICP[4-9]. ICP has been linked to adverse maternal and fetal outcomes. The main symptom is pruritus without evidence of skin lesions, which appears most typically in the third trimester of pregnancy. Laboratory tests demonstrate an increase in serum bile acids and aminotransferases[2,10,11]. ICP is essentially benign in mothers. The major consequences of this disease are premature delivery in 19%-60% of cases[12,13], stillbirths in 1%-2%[13,14] and fetal distress in 22%-33%[15,16]. The mechanisms by which ICP leads to poor fetal outcome are unclear. Recent clinical and biochemical studies have provided evidence for altered metabolism of bile acids and progesterone in ICP, although it remains unclear whether these changes are specific for ICP or are rather the consequence of cholestatic injury[17-20]. In a study from Sweden, a correlation between fetal complications and serum bile acids levels was demonstrated[3].

Various strategies have been proposed to improve obstetric outcome. Nevertheless, in several studies, the investigators have concluded that fetal death in ICP may not be predictable by traditional antepartum surveillance, and that delivery after establishment of fetal lung maturity may reduce fetal mortality rate[13-15]. Obstetric management consists of weighing the risk of premature delivery against the risk of sudden death *in utero*. As well, it has to be considered that induction of labor is associated with a higher frequency of complications such as surgical delivery compared to spontaneous labor[2]. To allow term delivery (≥ 37 wk) in patients with ICP, it appears essential to ascertain early prognostic markers for poor fetal outcome.

In our previous prospective therapeutic trial[21], we observed a significant effect of ursodeoxycholic acid (UDCA) in comparison to cholestyramine on pruritus, serum liver tests, and the duration of pregnancy in patients with ICP. However, the treatment groups did not differ significantly in the number of premature deliveries (< 37 wk gestation). Therefore, the aim of the current study was to re-evaluate clinical symptoms and biochemical parameters as potential predictors of spontaneous preterm births in patients with ICP.

MATERIALS AND METHODS

Sixty patients with ICP defined by (1) development of pruritus during the second or third trimester of pregnancy, and (2) total fasting serum bile acids (TBA) ≥ 11 μmol/L, were included in this retrospective analysis. All patients were seen at the Kaunas Medical University Hospital, Lithuania between October 1999 and September 2002. Patients with chronic liver diseases, skin diseases, allergic disorders, symptomatic cholelithiasis, and ongoing viral infections affecting the liver (hepatitis A, B and C virus, cytomegalovirus, herpes simplex virus, and Epstein-Barr virus) were excluded. All patients participated in a randomized parallel-group study as reported previously[21]. In contrast to the previous prospective trial, the actual retrospective analysis included patients with elevated TBA ≥ 11 μmol/L only.

Pruritus intensity was assessed daily by patients using a subjective score: 0, no pruritus; 1, mild pruritus, occasional; 2, moderate pruritus, intermittent during the day with asymptomatic periods prevailing; 3, severe pruritus every day with symptomatic periods prevailing; 4, severe, constant pruritus day and night. Serum liver tests and fasting serum bile acids were evaluated at the time of the first presentation. Serum liver tests were determined using routine laboratory techniques. Bile acids were analyzed by gas-liquid chromatography as described previously[21,22]. Fasting serum samples were stored at -20℃ until analyzed. Ultrasonography of the abdomen and serology of viral hepatitis were performed to exclude other causes of liver disease in every patient before enrollment.

The Obstetric and Gynecology Clinic of Kaunas Medical University Hospital is a tertiary care maternity center that provides all obstetric services for women with complicated pregnancies, for a stable and ethnically uniform population of 2 million inhabitants. Most of the high-risk deliveries in the area took place in this clinic. Fetal status was monitored in the same hospital every week. Pregnancy outcome and newborn status (term and mode of delivery, Apgar score at 1 and 5 min, asphyxial events, and newborn weight) were assessed by obstetricians and neonatologists, who were not given any specific instructions concerning date and form of delivery. Spontaneous preterm birth was defined as delivery before 37 wk gestation after the spontaneous onset of labor.

Statistical analysis

The results are expressed as means ± SD. Comparison of parametric, normally distributed data was performed by Student's *t* test. The difference between two samples was calculated using the Mann-Whitney test. Correlation analysis was assessed by Spearman's rank correlation. Multivariate analysis of significant prognostic factors of delivery before 37 wk gestation was based on binary multivariate logistic regression analysis. Factors found to be significant or having a trend towards significance (TBA concentrations, onset of pruritus) were selected for this model. Statistical analysis was conducted with SPSS 12.0. All reported *P* values were two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Sixty patients who met the inclusion criteria were included in the study. Age ranged between 18 and 40 year (median 27.0), median gestational age was 35.0 wk (range, 22-39), median time of onset of pruritus was 32.0 wk (range, 20-37). Twenty-eight (46.6%) women were primiparous and 32 (53.4%) were multiparous. Recurrence of ICP was reported by 19 (31.6%) patients, 14 of these had a history of preterm delivery, and two of intrauterine fetal death. Ten (16.6%) patients had been users of oral contraceptives, of whom three women had experienced pruritus during use. Gallstone disease was diagnosed in seven (11.6%) cases. One (1.6%) patient had a urinary tract infection. No stillbirths were observed. The Apgar score at 1 min was 8.5 ± 0.7, and at 5 min, 9.0 ± 0.6. Delivery was after 38.1 ± 1.7 wk. Postnatal development was normal in all babies. Pregnancy ended prematurely in eight (13.3%) patients: in three receiving UDCA and in five treated with cholestyramine. Table 1 compares the clinical characteristics of patients who had deliveries before 37 wk and those who had delivery after 37 wk gestation. Significantly higher levels of TBA (47.8 ± 15.2 *vs* 41.0 ± 10.0 μmol/L, $P < 0.05$), and a tendency towards earlier onset of pruritus (29.0 ± 3.9 *vs* 31.6 ± 3.3 wk, $P = 0.057$) were found in cases of premature delivery when compared with term delivery. The correlation coefficient between TBA levels and pruritus scores tended to be higher in patients with preterm delivery (0.733) when compared to those with term delivery (0.523). In cases of preterm delivery (< 37 wk gestation), TBA concentration correlated positively with onset of pruritus ($r = 0.678$), bilirubin ($r = 0.538$), alanine aminotransferase ($r = 0.343$), and aspartate aminotransferase ($r = 0.308$), whereas correlation was weaker in term delivery.

To unravel potential factors that may affect the time of delivery, we instituted a binary multivariate logistic

Table 1 Clinical characteristics at the time of first presentation of patients with ICP who had delivery before and after 37 wk gestation

Characteristics	Delivery before 37 wk *n* = 8	Delivery after 37 wk *n* = 52	*P*
Age (yr)	26.6 ± 7.2	28.3 ± 5.4	0.433
Onset of pruritus (wk)	29.0 ± 3.9	31.6 ± 3.3	0.057
Intensity of pruritus (score)	3.1 ± 0.4	2.9 ± 0.6	0.361
ALT (U/L)	187.3 ± 87.2	210.4 ± 149.3	0.721
AST (U/L)	126.6 ± 78.8	140.1 ± 101.4	0.855
AP (U/L)	386.9 ± 132.7	372.1 ± 136.2	0.707
γGT (U/L)	35.6 ± 23.2	24.4 ± 14.1	0.072
Bilirubin (μmol/L)	10.3 ± 4.0	15.5 ± 13.1	0.202
TBA before treatment (μmol/L)	47.8 ± 15.2	41.0 ± 10.0	0.041

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphotase; γGT, γ-glutamyltransferase; TBA, total bile acids.

Table 2 Potential risk factors for preterm delivery: Predictive value as evaluated by binary multivariate logistic regression analysis

Factor	OR	95.0% CI	*P*
Onset of pruritus (wk)	1.703	1.227-2.947	0.038
Total bile acids before treatment (μmol/L)	2.128	1.126-3.252	0.013

regression model. The factors that were found to be significant or those that had a trend towards significance (TBA concentrations, onset of pruritus) were selected for this model. Binary multivariate logistic regression analysis demonstrated that serum bile acid concentration (OR 2.13, 95% CI 1.13-3.25, $P = 0.013$) and onset of pruritus (OR 1.70, 95% CI 1.23-2.95, $P = 0.038$) were the most important independent variables predicting preterm delivery (Table 2).

DISCUSSION

Although recent studies have improved our understanding of the underlying pathophysiological disturbances and their association with specific symptoms during ICP, the pathogenesis and prognosis of pregnancy have remained obscure. The current study aimed to unravel potential risk factors for fetal prematurity. We found that earlier onset of pruritus and higher TBA concentrations were associated with preterm delivery in our cohort of patients with ICP. The correlation between premature delivery and onset of pruritus is a new and interesting finding; although, high TBA levels have already been described as predictors of fetal outcome in other cohorts[3,23].

ICP is the most common liver disorder unique to pregnancy. In Lithuania, a retrospective analysis disclosed a rate of 0.4% of ICP in 16252 pregnant women over a period of 5 year (1996-2000; J. Kondrackiene, unpublished data). Although essentially benign in the mother, ICP may adversely affect the prognosis of the fetus. ICP has been reported to be associated with increased rates of spontaneous premature delivery[12,13]. According to the Lithuanian Medical Birth Register (2004), the total premature birth rate was 5.3%. Our study showed a 13.3% incidence of preterm delivery in patients with ICP.

The mechanism of preterm delivery remains unclear. Germain *et al*[24] have shown that during ICP, activation of the oxytocin receptor pathway is possibly caused by a cholic-acid-mediated increase in oxytocin-receptor expression. The placenta plays a crucial role in protecting the fetus from the adverse effects of potentially toxic endogenous substances, including TBA[25]. High levels of maternal TBA affect placental transport, placental hormone production, and chorionic vessel constriction[17]. In animal models, maternal hypercholanemia may affect the vectorial transfer of bile acids through the creation of inversely directed gradients, as compared with the physiological situation[26], and by impairing the ability of the trophoblast to transport bile acids[18]. A study from Argentina has shown that asymptomatic hypercholanemia of pregnancy, defined as TBA > 11 μmol/L in healthy pregnant women, does not necessarily lead to ICP[27]. Glantz *et al*[3] have demonstrated that no increase in fetal risk is detected in ICP patients with TBA levels < 40 μmol/L, and have proposed that these women can be managed expectantly. However, a recent case of fetal death at 39 wk and 3 d in a patient with ICP, who had low TBA concentrations at the time of diagnosis, has been reported[28]. This raises a crucial question: is the fasting TBA level sufficient to predict fetal outcome? Should testing be repeated on a weekly basis or discontinued once a less-than-critical level has been determined? What if concentrations increase dramatically over the following weeks, although levels are comparably low at the time of diagnosis for which the prognostic value has been evaluated[29]? Therefore, it is important to evaluate other clinical factors that are possibly associated with prematurity. We found significantly higher levels of TBA, and a tendency towards earlier onset of pruritus, although non-significant, in patients who had premature delivery, when compared with cases of term delivery. The binary multivariate regression analysis revealed that the TBA levels and early onset of pruritus were the most important independent factors predicting premature delivery.

In the current retrospective study, we did not analyze the effect of treatment on preterm delivery. Indeed, among the women who had births before 37 wk gestation, three of eight patients had received UDCA, and five were treated with cholestyramine. The size of the cohort may have been too small to detect any difference in the rate of preterm delivery between patients treated with UDCA and those treated with cholestyramine, although the timepoint of delivery was significantly earlier in patients treated with cholestyramine than in those treated with UDCA in our previous analysis. As well, the rate of preterm delivery in the present cohort was lower than that reported in other studies. This could in part be due to increased attention devoted to ICP during the study[3].

The current analysis indicated that early onset of pruritus, along with markedly elevation of TBA levels, may predict premature delivery, which represents a potential risk factor for the fetus in women with ICP. Because the

prognosis remains unpredictable in some cases[28], our current strategy is to begin pharmacological treatment after confirmation of diagnosis in all ICP patients. The treatment of choice is UDCA, which has improved maternal and fetal morbidity in several clinical trials and observational studies[21,31-34]. When lung maturity is achieved for those patients with risk factors of prematurity, delivery should be considered.

In conclusion, the present study indicated that early onset of pruritus and high levels of TBA were the most important factors associated with preterm delivery in a well-defined cohort of patients with ICP; thereby, defining a group at risk of poor neonatal outcome and so requiring active management.

COMMENTS

Background

Intrahepatic cholestasis of pregnancy (ICP) is characterized by pruritus and an elevation in serum bile acid concentrations. The major consequences of this disease are premature delivery, stillbirth and fetal distress. The mechanisms by which ICP leads to poor fetal outcome are unclear, although a role for bile acids or toxic metabolites of bile acids has been suggested. Currently, the hydrophilic bile acid ursodeoxycholic acid (UDCA) is the most effective treatment for ICP. Various strategies have been proposed to improve obstetric outcome. In several studies, the investigators have concluded that fetal death in ICP may not be predictable by traditional antepartum surveillance, and that delivery after establishment of fetal lung maturity may reduce fetal mortality rate. To allow for term delivery ($\geqslant$ 37 wk) in patients with ICP, it appears essential to disclose early prognostic markers for a poor fetal prognosis.

Research frontiers

There is increasing evidence that genetically determined dysfunction in the canalicular ABC transporters might be risk factors for development of ICP. Heterozygous mutations in the MDR3 gene (encoding for a canalicular phospholipid translocator involved in the biliary secretion of phospholipids) have been found. Recent clinical and biochemical studies provided evidence of abnormal metabolites impairing hepatobiliary carriers for an altered metabolism of bile acids and progesterone in ICP although it remains unclear whether these changes are specific for ICP or are rather the consequence of cholestatic injury in ICP.

Innovations and breakthroughs

We found that earlier onset of pruritus and higher fasting serum bile acid concentrations were associated with preterm delivery in our cohort of patients with ICP. The correlation between premature delivery and onset of pruritus is a new and interesting finding; although, high serum bile acid levels have been described as predictors of fetal outcome in other cohorts.

Applications

The present study indicates that early onset of pruritus and high levels of serum bile acid are the most important factors associated with preterm delivery in patients with ICP; thereby, defining a group at risk of poor neonatal outcome and so requiring active management.

Peer review

This is a well-written manuscript reporting on a cohort of 60 patients with symptomatic ICP. Early onset of pruritus and high levels of serum bile acids predict preterm delivery in intrahepatic cholestasis of pregnancy and define a subgroup of patients at risk of poor neonatal outcome.

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RAPID COMMUNICATION

Bevacizumab plus infusional 5-fluorouracil, leucovorin and irinotecan for advanced colorectal cancer that progressed after oxaliplatin and irinotecan chemotherapy: A pilot study

Hyuk-Chan Kwon, Sung Yong Oh, Suee Lee, Sung-Hyun Kim, Hyo-Jin Kim

Hyuk-Chan Kwon, Sung Yong Oh, Suee Lee, Sung-Hyun Kim, Hyo-Jin Kim, Departments of Internal Medicine, Dong-A University College of Medicine, Busan 602-715, Korea
Correspondence to: Hyo-Jin Kim, MD, PhD, Department of Internal Medicine, Dong-A University College of Medicine, 3-1 Dongdaeshin-dong, Seo-gu, Busan 602-715, Korea. kimhj@dau.ac.kr
Telephone: +82-51-2402951 Fax: +82-51-2402088
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Abstract

AIM: To evaluate the combination of bevacizumab with infusional 5-fluorouracil (5-FU), leucovorin (LV) and irinotecan (FOLFIRI) in patients with advanced colorectal cancer (CRC) pretreated with combination regimens including irinotecan and oxaliplatin.

METHODS: Fourteen patients (median age 56 years) with advanced CRC, all having progressed after oxaliplatin- and irinotecan-based combination chemotherapy, were enrolled in this study. Patients were treated with 2 h infusion of irinotecan 150 mg/m^2 on d 1, plus bevacizumab 5 mg/kg iv infusion for 90 min on d 2, and iv injection of LV 20 mg/m^2 followed by a bolus of 5-FU 400 mg/m^2 and then 22 h continuous infusion of 600 mg/m^2 given on two consecutive days every 14 d.

RESULTS: The median number of cycles of chemotherapy was six (range 3-12). The response rate was 28.5%, one patient had a complete response, and three patients had a partial response. Eight patients had stable disease. The median time to progression was 3.9 mo (95% CI 2.0-8.7), and the median overall survival was 10.9 mo (95% CI 9.6-12.1). Grade 3/4 neutropenia occurred in five patients, and two of these developed neutropenic fever. Grade 3 hematuria and hematochezia occurred in one. Grade 2 proteinuria occurred in two patients. However, hypertension, bowel perforation or thromboembolic events did not occur in a total of 90 cycles.

CONCLUSION: Bevacizumab with FOLFIRI is well tolerated and a feasible treatment in patients with heavily treated advanced CRC.

Key words: Bevacizumab; Irinotecan; Leucovorin; 5-fluorouracil; Colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer deaths worldwide, with 945 000 new cases and 492 000 CRC-related deaths in 2000[1,2]. Up to 30% of patients present with metastatic disease, and approximately 50%-60% eventually develop metastatic or advanced disease[1]. The management of patients with metastatic CRC has changed dramatically over the last 5 years, with increased chances of prolonged survival. In particular, combination chemotherapy regimens including irinotecan and oxaliplatin have markedly improved response rates and prolonged median survival over fluorouracil (FU) with leucovorin (LV)[3,4]. However, there is a paucity of data about third-line chemotherapy in patients who are resistant to irinotecan- or oxaliplatin-based chemotherapy[5-7].

Angiogenesis is required for tumor growth and metastasis, which makes it an attractive target for biologically based cancer therapy[8-10]. Vascular endothelial growth factor (VEGF) is the most potent and specific target for cancer therapy, and has been identified as a crucial regulator of both normal and pathological angiogenesis, with increased expression being observed in many human tumor types[11-13]. In CRC, increased VEGF expression correlates with invasiveness, vascular density, metastasis, recurrence and prognosis[14,15]. In preclinical studies, a murine anti-human monoclonal antibody directed against VEGF has been shown to inhibit the growth of human tumor xenografts[16-18]. As well, the combination of anti-VEGF antibody and chemotherapy in nude mice injected with human cancer xenografts has demonstrated an increased antitumor effect compared with antibody or chemotherapy treatment alone[19].

Bevacizumab, a recombinant humanized monoclonal antibody targeting VEGF, has been evaluated in various solid tumors[20]. In phase I trials, bevacizumab was generally well tolerated and did not demonstrate dose-limiting toxicity or interactions with commonly used chemotherapy

regimens[21]. In a phase 2 trial of treatment of CRC, the addition of bevacizumab to FU/LV increased the response rate, the median time to disease progression, and the median duration of survival[22]. Recently, it has been shown in randomized phase Ⅲ trials that bevacizumab, when combined with irinotecan plus bolus FU/LV in the first-line treatment of metastatic CRC, and with oxaliplatin plus continuous FU/LV (FOLFOX) in second-line treatment leads to an increased median survival, progression-free survival (PFS), and response rate compared with cytotoxic chemotherapy alone[23,24].

The goal of this trial was to evaluate the safety and activity of bevacizumab plus LV, 5-FU and irinotecan (FOLFIRI) in patients with advanced CRC that had progressed after treatment with both irinotecan- and oxaliplatin-based chemotherapy regimens.

MATERIALS AND METHODS

Eligibility criteria

The eligibility criteria were as follows: histologically confirmed CRC (adenocarcinoma), bidimensionally measurable disease, no secondary malignancy, age > 18 years, Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0-2, and a life expectancy of > 3 mo. Adequate hematological, hepatic and renal function (including urinary excretion of no more than 500 mg protein/d) were also required. Patients should have had and failed both oxaliplatin- and irinotecan-based treatment prior to enrolment. Failure due to significant intolerance to either drug was allowed.

Exclusion criteria included thromboembolism that required therapeutic anticoagulation, central nervous system metastasis, and major surgery within 6 wk, non-healing wounds, uncontrolled hypertension, pregnant or lactating women, bleeding diathesis, active or recent cardiovascular disease or cerebrovascular accident, or prior bevacizumab therapy. The pretreatment characteristics of the patients are presented in Table 1. Written informed consent was required before chemotherapy.

Table 1 Patient characteristics

Characteristics	No. of patients
Median age (range)	56 yr (29-69)
Sex	
Male	9
Female	5
Performance (ECOG)	
0-1	12
2	2
CEA (ng/mL)	
< 5	3
⩾ 5	11
Primary site	
Colon	8
Rectum	6
Sites of metastasis	
Liver	7
Lung	8
Lymph nodes	7
Peritoneum	3
Others	2
Number of metastasis	
1	3
⩾ 2	11
Adjuvant chemotherapy	
Yes	9
No	5

CEA: Carcinoembryonic antigen.

Treatment protocols and dose modification

On d 1, irinotecan (150 mg/m^2) was administered in 500 mL normal saline or dextrose as a 2-h iv infusion. On d 1 and 2, LV (20 mg/m^2) was administered as a iv bolus, immediately followed by 5-FU (400 mg/m^2) given as a 10-min iv bolus, followed by 5-FU (600 mg/m^2) as a continuous 22-h infusion. Bevacizumab administration always followed chemotherapy. Bevacizumab was given at 5 mg/kg as an iv infusion every 2 wk. The first infusion was given over 90 min, the second over 60 min, and if both were well tolerated, subsequent infusions were given over 30 min. No premedication was given.

Dose modifications of irinotecan or 5-FU were made for hematological or non-hematological toxicity, on the basis of the most severe grade of toxicity that occurred during the previous cycle. Treatment was delayed until the absolute number of neutrophils was > 1500/μL, platelets were > 100000/μL, and recovery occurred from mucositis, diarrhea, or skin toxicity to grade 1 or less. The 5-FU dose was reduced after the occurrence of National Cancer Institute Common Toxicity Criteria (NCI-CTC) grade 3 diarrhea, stomatitis or dermatitis. For toxicity of grade 3 or higher, a dose reduction of irinotecan by 20% was prescribed by the protocol. Bevacizumab was retained for uncontrolled hypertension or proteinuria of > 2 g in 24 h. Bevacizumab was discontinued for grade 3 or 4 hemorrhage, thromboembolic events that required full-dose anticoagulation, or any grade 4 toxicity.

Treatment was administered until the disease progressed, unacceptable toxic effects developed, or the patient refused further treatment.

Pretreatment and follow-up evaluation

Pretreatment evaluation included physical examination, complete blood cell counts, blood chemistry, tumor marker level, and radiological examination [chest posterior-anterior (PA) view radiography, computed tomography (CT) and other imaging techniques as clinically indicated] within 1 mo of starting chemotherapy. Tumor responses were determined by WHO criteria[25]. Complete blood cell counts, serum chemistry, including liver and renal function, and chest PA radiography were performed at least every 2 wk, and tumor assessment by CT was performed every three cycles.

Statistical analysis

Efficacy analysis was performed according to the intention-to-treat principle. Patients were considered assessable for response if they were eligible, had measurable disease, and had received at least one dose of study therapy. In the analysis of survival and subsequent treatment, all patients

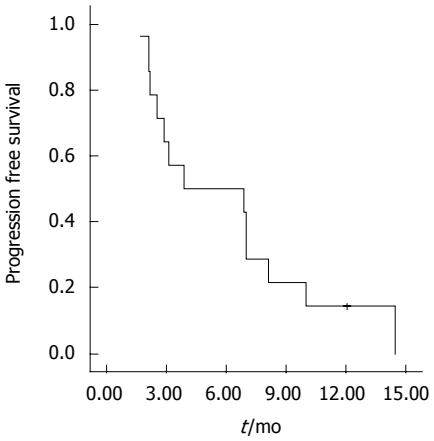

Figure 1 PFS curve. Median PFS was 3.9 mo (95% CI: 2.0-8.7).

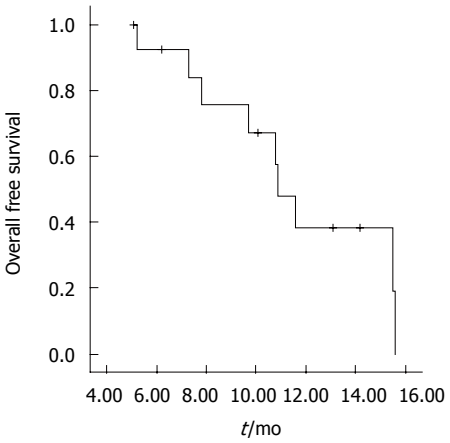

Figure 2 OS curve. Median OS was 10.9 mo (95% CI: 9.6-12.1).

were followed until death, loss to follow-up, or termination of the study.

PFS and overall survival (OS) were calculated using the Kaplan-Meier method. PFS was calculated from the date therapy started to the date of disease progression, and OS was calculated from the date therapy started to the date of death. All data were analyzed using SPSS software (version 12.0, Chicago, IL, USA).

RESULTS

Patient characteristics

Between June 2005 and June 2006, a total of 14 patients were assigned for treatment at the Department of Internal Medicine, Dong-A University Medical Center, Busan, Korea. Demographic details of the patients included in the study are shown in Table 1. There were nine male and five female patients, median age 59 years (range 29-69). All patients had progressed after prior irinotecan- and oxaliplatin-based regimens. All patients used bevacizumab/FOLFIRI treatment within 6 mo after both irinotecan and oxaliplatin treatment failures. All 14 patients were assessable for response and for toxicity and survival.

Objective tumor responses and survival

There were a median six cycles of chemotherapy (range 3-12). Chemotherapy was stopped due to disease progression in 12 patients, one discontinued because of toxicity and another because of unrelated events with no progression. Median follow-up duration was 10.1 mo. Response rate was 28.5% (95% confidence interval (CI) 15.0%-36.5%), one patient had a complete response, and three had a partial response. The median duration of response was 7.7 mo. Eight patients had stable disease, and two had disease progression. PFS was 3.9 mo (95% CI; 2.0-8.7), and median OS was 10.9 mo (95% CI; 9.6-12.1). Figures 1 and 2 show PFS and OS curves, respectively. Oral 5-FU was administered to the 12 patients with disease progression. No objective responses were documented after salvage therapy.

Table 2 Toxicity (n = 14)

	NCI-CTC grade			
	1	2	3	4
Hematological				
Anemia	7	3		
Leukopenia	2	2	2	
Neutropenia		3	4	1
Thrombocytopenia	1	3	1	
Non-hematological				
Nausea/vomiting	2	1		
Mucositis	1			
Diarrhea		2		
Proteinuria	1	2		
Hematuria	1		1	
Asthenia		4		

Toxicity

A total of 90 cycles of chemotherapy were administered to the patients. All patients who received at least one dose of bevacizumab and chemotherapy were assessable for adverse events. Dose modifications or interruptions were required in 30% of patients. The incidence of hematological and non-hematological toxicity is summarized in Table 2. The major grade 3/4 hematological toxicity included neutropenia (35.7%) and thrombocytopenia (7.1%). There were two cycles of neutropenic fever. Grade 1/2 nausea, vomiting, diarrhea and mucositis developed in 6 patients; however, this toxicity was mild and manageable. In one patient who had rectal cancer with bladder invasion, massive hematuria and hematochezia occurred; he was taken off the study because of toxicity. However, hypertension, bowel perforation or thromboembolic events did not occur. There was no treatment-related death. There have been nine reported deaths, of which eight were because of disease progression, and one was because of pneumonia in the present study.

DISCUSSION

Patients with advanced CRC treated with 5-FU, irinotecan and oxaliplatin in combination or sequentially may survive for 18-21 mo[3,4,26,27]. However, if these three standard drugs fail, there are no accepted treatment options. There have been few clinical trials in a third-line setting that can

provide historical estimates of PFS and OS[5-7,28,29]. A recent study has shown that patients treated with cetuximab in combination with irinotecan achieved significant activity[6]. The response rate was 22.9% and time to progression and OS were 4.1 and 8.6 mo, respectively. Promising data from a small randomized phase Ⅱ trial have recently shown that bevacizumab when added to cetuximab or to cetuximab plus irinotecan has a high activity in chemotherapy-refractory CRC[28]. Panitumumab, a human monoclonal antibody against epidermal growth factor receptor (EGFR), has also been shown to be active in irinotecan- and oxaliplatin-refractory metastatic CRC[23]. However, other reports have shown no clinical benefits[5,7].

The improvement in the clinical outcome afforded by the addition of bevacizumab to 5-FU suggests that blocking VEGF may be a broadly applicable approach to the treatment of CRC[22]. Adding bevacizumab to both first- and second-line combination chemotherapy improves response, time to progression, and OS, but not without toxicity[23,24]. The addition of bevacizumab 5 mg/kg biweekly significantly improved the primary outcome of median survival from 15.6 mo with irinotecan/5-FU bolus infusion/LV (IFL) alone to 20.3 mo with IFL/bevacizumab. Bevacizumab also significantly increased response rate from 34.8% to 44.8%, and prolonged time to progression from 6.2 to 10.6 mo[23]. Compliance was also excellent in this study. As well, results from a phase Ⅲ study in patients with previously treated metastatic colon cancer have revealed improved OS in patients who receive bevacizumab (10 mg/kg) with FOLFOX, as compared with those treated with FOLFOX alone, 12.5 versus 10.7 mo[24].

However, a recent large non-randomized study has shown that the combination of bevacizumab and a bolus regimen of 5-FU/LV is not sufficiently active in heavily pretreated, bevacizumab-naive patients to support the use of bevacizumab with bolus 5-FU/LV in chemotherapy-refractory metastatic CRC. The combination of bevacizumab and 5-FU/LV was associated with a low response rate: 4% based on investigator assessment and 1% based on independent review. Median PFS and OS were 3.7 and 9.1 mo, respectively[7]. This study demonstrated that for patients with advanced CRC that had progressed after treatment with both oxaliplatin- and irinotecan-based chemotherapy regimens, response rate was 28.5%, with approximately 58% of the patients showing stable disease. Median PFS was 3.9 mo and median OS was 10.9 mo. We used irinotecan instead of bolus 5-FU/LV; therefore, the response rate and survival were increased compared with those in the earlier study. Further studies will be needed to confirm these results.

Previous phase 1 and 2 clinical trials have suggested that treatment with bevacizumab alone or with chemotherapy results in an increased incidence of thrombosis, bleeding, proteinuria and hypertension[21,22]. In two phase Ⅲ investigations, the risk of venous thromboembolism was not increased by bevacizumab, but there was a small increased risk of both bleeding and bowel perforations, as well as a consistent increase in hypertension[23,24]. Hemorrhage has also been seen more frequently with bevacizumab treatment as compared with chemotherapy alone[22]. The majority of patients had minor hemorrhage, but 10% of patients had gastrointestinal hemorrhage, and 43% were grade 3/4. However, a larger phase Ⅲ trial did not demonstrate an increased incidence of grade 3/4 bleeding[23]. We did not find any excess of such side effects, compared with previous studies, except for one case of massive bleeding. The reason why there was no thrombosis, hypertension and bowel perforation may have been due to the small number of patients and their relatively young age.

An analysis of predictive markers has shown indeed that bevacizumab increases the activity of irinotecan plus FU/LV, regardless of the level of VEGF expression, thrombospondin expression, and microvessel density[30]. In this study, we evaluated the correlation between expression of VEGF and microvascular density and clinical outcome, and we found no significant results (data not shown).

COMMENTS

Background

There is a paucity of data about third-line chemotherapy in patients who are resistant to irinotecan- or oxaliplatin-based chemotherapy. Bevacizumab, a recombinant humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF), has been evaluated in various solid tumors.

Research frontiers

The goal of this trial was to evaluate the safety and activity of bevacizumab plus irinotecan (FOLFIRI) in patients with advanced colorectal cancer (CRC) that had progressed after treatment with both irinotecan- and oxaliplatin-based chemotherapy regimens.

Innovations and breakthroughs

Bevacizumab with FOLFIRI is well tolerated and feasible in heavily treated patients with advanced CRC.

Applications

We will evaluate correlations between expression of VEGF and microvascular density and clinical outcomes.

Terminology

Bevacizumab: monoclonal antibody against VEGF, which aids growth and metastasis of several cancers. Irinotecan, oxaliplatin: chemotherapeutic agents that are useful in CRC.

Peer review

The manuscript evaluates the safety and activity of bevacizumab plus FOLFIRI in patients with advanced CRC that had progressed after treatment with both irinotecan- and oxaliplatin-based chemotherapy regimens. The method is simple and correct. The manuscript is written in correct English with minor language polishing.

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RAPID COMMUNICATION

Predictive factors for interferon and ribavirin combination therapy in patients with chronic hepatitis C

Joonho Yoon, Jong In Lee, Soon Koo Baik, Kwang Ho Lee, Joon Hyung Sohn, Hyean-Woo Lee, Jun Namkung, Sei Jin Chang, Jong-Whan Choi, Hyun-Won Kim, Byung-Il Yeh

Joonho Yoon, Joon Hyung Sohn, Hyean-Woo Lee, Jun Namkung, Jong-Whan Choi, Hyun-Won Kim, Byung-Il Yeh, Department of Biochemistry and Institute of Basic Medical Sciences, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Jong In Lee, Soon Koo Baik, Department of Internal Medicine, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Kwang Ho Lee, Department of Anesthesiology, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Sei Jin Chang, Department of Preventive Medicine and Institute of Occupational Medicine, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Soon Koo Baik, Jun Namkung, Sei Jin Chang, Byung-Il Yeh, Institute of Lifelong Health, Wonju College of Medicine, Yonsei University, Wonju 220-701, Republic of Korea
Supported by a Research Grant from Yonsei University, Wonju College of Medicine, YUWCM 2004-03
Correspondence to: Byung-Il Yeh, Department of Biochemistry, Wonju College of Medicine, Yonsei University, 162 Ilsandong, Wonju, Gangwondo 220-701,
Republic of Korea. biyeh64@hotmail.com
Telephone: +82-33-7410282 Fax: +82-33-7430411
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Abstract

AIM: To confirm the predictive factors for interferon (IFN)-α and ribavirin combination therapy for chronic hepatitis patients with hepatitis C virus (HCV) genotype 1b.

METHODS: HCV RNA from 50 patients infected with HCV genotype 1b was studied by cloning and sequencing of interferon sensitivity determining region (ISDR), PKR-eIF2α phosphorylation homology domain (PePHD). Patients were treated with IFN-α and ribavirin for 6 mo and grouped by effectiveness of the therapy. A variety of factors were analyzed.

RESULTS: Our data showed that age, HCV RNA titer, and ISDR type could be used as the predictive factors for combined IFN-α and ribavirin efficacy. Characteristically, mutations in PePHD appeared only when the combination therapy was effective. Other factors, such as sex and alanine aminotransferase (ALT) level, were not related to its efficacy. Adjusting for age and HCV RNA titer indicated that the ISDR type was the most potent predictive factor.

CONCLUSION: HCV RNA ISDR type is an important factor for predicting efficacy of IFN-α and ribavirin combination therapy in Korean patients.

Key words: Hepatitis C virus -1b; Interferon-α; Ribavirin; Interferon sensitivity determining region; PKR-eIF2α phosphorylation homology domain

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped RNA virus with a positive single-stranded RNA genome about 9600 nucleotides in length. This RNA encodes a single polypeptide approximately 3000 amino acids in length. The polypeptide is post-translationally cleaved into structural and non-structural proteins[1-3]. HCV infection is a major cause for chronic liver disease worldwide[4]. Eighty percent or more of acutely infected patients develop chronic hepatitis, which progresses to liver cirrhosis in about 20% of cases and hepatocellular carcinoma in 5%. These complications arise even if the disease remains asymptomatic[5].

Alpha-interferon (IFN-α) treatment effectively reduces viral load, but complete eradication of the virus is achieved in less than 20% of patients treated with IFN-α alone and in 40%-47% of patients treated with combined IFN-α and ribavirin[6-8]. The treatment outcome largely depends on the sensitivity of HCV genotype and IFN-α[9,10]. About 10 years before, IFN-α and ribavirin combination therapy has begun to be used instead of IFN monotherapy. Ribavirin is an oral nucleoside analogue acting on a broad spectrum of DNA and RNA viruses. It has been proposed to have both direct antiviral and immunomodulatory effects[11], but the detailed mechanism remains unclear. Ribavirin used as monotherapy is known to have little or no activity against HCV.

The HCV genotype appears to be a major determinant for IFN efficacy, because patients infected with HCV genotypes 2 and 3 respond better to IFN monotherapy than patients with genotype 1[9]. The IFN-based therapy effectiveness is not satisfactory, especially in patients with HCV genotype 1b[12-14], the most common genotype in Korea[15,16], Japan[17], southern and eastern Europe[18,19]. IFN is also inconvenient to use, costly, and has a variety

of possible complications. Thus, many studies have been carried out to determine the predictive factors for the efficacy IFN therapy[12-19].

Response to interferon monotherapy is associated with several host and viral factors. HCV genotype 1b, low viral load, and rapid HCV RNA clearance from the serum have been identified as favorable predictors for a sustained response to IFN therapy[20-22]. Since Enomoto *et al*[23] reported that genetic variability in a 40 amino acid stretch (amino acids 2209-2248) and mutations in the NS5A region of HCV and in designated interferon sensitivity determining region (ISDR), has become a predictive factor for IFN therapy. Studies from Japan[23], Sweden[24] and Spain[25] have shown that ISDR is an effective predictor. However, studies from Western countries displayed that ISDR is not a good predictor[26-28]. Studies on the relationship between IFN-α and ribavirin combination therapy and ISDR have controversial results[29-32].

Taylor *et al*[31] reported that a HCV envelope protein (E2) contains a sequence similar to the phosphorylation site on eIF2-α for the interferon-inducible cellular protein kinase PKR. The PKR-eIF2-α phosphorylation homology domain (PePHD) on E2 may serve as a pseudosubstrate for PKR and inhibit its function, reducing the antiviral effect of interferon. Thus, the PePHD region might also be involved in IFN resistance of chronic hepatitis C to IFN therapy. However, the role of this region is also controversial so far[31].

In Korea, the HCV prevalence is about 1%-2%, but studies to analyze the predictive factors for combined IFN-α and ribavirin therapy have not been performed. To identify these factors, we investigated the relationship between combined IFN-α and ribavirin efficacy and a variety of factors such as ISDR sequence, PePHD sequence, age, ALT level, and HCV RNA titer in Korean patients with HCV genotype 1b.

MATERIALS AND METHODS

Patents and treatment

Serum was collected from HCV genotype 1b-infected patients admitted to Wonju Christian Hospital. Only HCV genotype 1b was used in this study because it is the most common HCV genotype in the Republic of Korea. Sera were screened by a third generation ELISA method with an anti-HCV antibody. The patients were treated with IFN-α and ribavirin for 6 mo. Three million units of IFN-α was injected every two days, and 9 mg of ribavirin was orally administrated during the same period. The patients who did not receive the treatment were excluded. After the 6-mo combination therapy, the patients were classified into complete response group and no-response group. In the complete response group, HCV RNA titer was less than 50 IU/mL and ALT levels were within the normal range. In the no-response group, HCV RNA titer was over 50 IU/mL even if the ALT levels were normal.

cDNA preparation

HCV RNA was extracted from sera as previously described[33]. After ethanol precipitation, each RNA pellet was dissolved in 10 μL of diethylpyrocarbonate (DEPC)-treated distilled water for cDNA preparation. cDNA synthesis was performed as previously described[34] with certain modifications. For the synthesis of cDNA of HCV, an aliquot of RNA (10 μL) isolated from the sera of patients was mixed with 1 μL of random hexamer (1 μmol/L), 2 μL of reaction buffer (250 mmol/L Tris-HCl pH 8.3, 250 mmol/L potassium chloride, 50 mmol/L magnesium chloride, 50 mmol/L dithiothreitol and 2.5 mmol/L spermidine) and 5.5 μL of DEPC-treated water was added. After the contents were heat-treated for 5 min at 65℃, 20 units (0.5 μL) of RNase inhibitor and 10 units (1 μL) of AMV reverse transcriptase were added. The mixture was incubated at 37℃ for 30 min, followed by at 99℃ for 1 min to inactivate the enzyme. PCR was performed as described previously[16]. The ISDR and PePHD primer sequences are listed in Table 1. PCR products were subjected to agarose gel electrophoresis in Tris-acetate-EDTA buffer and visualized with ethidium bromide staining under an ultraviolet transilluminator.

ISDR and PePHD sequencing

RT-PCR amplified products, including the ISDR and/or PePHD regions, were purified from agarose gel and glass milk (Gene Clean II kit, Bio 101, USA), and then subcloned by inserting the cDNA into a pGEM-T TA-cloning vector (Promega). The clones from each of the individual patient's plates were randomly selected and plasmid prepared from each clone was used as a template for DNA sequencing which was performed as previously described[35].

HCV RNA quantitation

In order to determine the HCV RNA titer, a quantitative and competitive polymerase chain reaction (QCPCR) assay was carried out as previously described[36]. As a first step, cDNA encoding the 5'-untranslated region of HCV was subcloned into a pGEM vector (pGEM5'UTR). Using PCR, the internal control plasmid, pGEM5'UTRDel, was constructed by deletion of nucleotides between the 87 and 165 nucleotides in the 5'-UTR of the HCV genome. The internal control RNA was synthesized *in vitro* by T7 RNA polymerase from a linearized template derived from the pGEM5'UTRDel plasmid. The amount of RNA synthesized *in vitro* was determined by measurement of the absorbance at 260 nm. A known copy number of the RNA was included as an internal control in order to quantify the viral RNA. The data were analyzed by Quantity One® 1-D analysis software (Bio-Rad).

Statistical analysis

Comparisons between groups were made by the Student's *t*-test. The *P* values were determined between the two groups with regard to age, ALT, amino acid mutations in PePHD, and HCV RNA titer. $P < 0.05$ was considered statistically significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for ISDR to test its predictive value for combination therapy by logistic regression analysis.

RESULTS

Patient characteristics

We collected serum from 50 HCV genotype 1b-infected

Table 1 Primer sequences used to amplify ISDR and PePHD

Region	Primer direction	Sequence (5′ to 3′)	Nucleotide No
ISDR	Outer sense	TGGATGGAGTGCGGTTGCACAGGTA	6703-6727
	Outer antisense	TGTAAAACGACGGCCAG	7296-7320
	Inner sense	TCTTTCTCCGTGGAGGTGGTATTGC	6722-6741
	Inner antisense	CAGGAAACAGCTATGACC	7275-7294
PePHD	Outer sense	TGACTACCCATACAGGCTCT	2180-2199
	Outer antisense	AAGGAAGGAGAGATTGCCAT	2725-2744
	Inner sense	AAGGTTAGGATGTATGTGGG	2238-2257
	Inner antisense	ATTGAGGACCACCGAGTTCT	2689-2708

ISDR: Interferon sensitivity determining region; PePHD: PKR-eIF2α phosphorylation homology domain; PKR: RNA-activated protein kinase.

Table 2 Characteristics of the complete response group patients before IFN-α and ribavirin combination therapy

	Age (yr)	Sex	ALT	Transfusion history[1]	HCV RNA titer[2]	ISDR Type[3]	No. of Amino Acid Mutations of PePHD
A-1	50	M	178	×	3.92	1	0
A-2	49	F	135	○	3.27	1	1
A-3	40	M	106	×	4.16	1	1
A-4	45	M	218	○	5.48	1	0
A-5	51	F	92	×	4.04	1	0
A-6	41	M	143	×	3.75	2	0
A-7	56	F	167	×	4.24	2	0
A-8	58	M	86	×	5.26	2	0
A-9	49	M	99	○	5.12	2	1
A-10	40	F	129	○	4.41	2	0
A-11	54	F	201	×	3.73	2	1
A-12	53	M	179	×	4.21	2	1
A-13	52	M	234	×	4.25	2	0
A-14	47	M	311	×	5.11	2	0
A-15	49	M	86	×	6.12	3	1
A-16	53	F	220	×	5.91	3	0
A-17	47	F	194	×	4.82	3	0
A-18	49	M	246	×	5.44	3	1
A-19	51	M	441	○	5.22	3	2
A-20	47	F	305	○	4.85	3	0
A-21	55	M	119	×	3.73	3	0

ISDR: Interferon sensitivity determining region; PePHD: PKR-eIF2α phosphorylation homology domain. [1]○ indicates history of transfusion and × indicates no history of transfusion. [2]The unit of HCV RNA titer before treatment (log copies/mL). [3]ISDR type: 1 (wild type, no amino acid substitution), 2 (intermediate type, 1-3 amino acid substitutions), 3 (mutant type, ⩾ 4 amino acid substitutions).

Table 3 Characteristics of the no-response group patients before IFN-α and ribavirin combination therapy

	Age (yr)	Sex	ALT	Transfusion history[1]	HCV RNA titer[2]	ISDR type[3]	No. of Amino Acid Mutations of PePHD
B-1	53	M	185	×	5.25	1	0
B-2	48	M	320	×	6.11	1	0
B-3	44	F	125	○	4.88	1	0
B-4	49	F	175	×	5.72	1	0
B-5	54	M	151	○	6.21	1	0
B-6	58	F	190	×	5.14	1	0
B-7	62	F	212	×	6.35	1	0
B-8	64	M	252	○	7.11	1	0
B-9	45	M	145	×	6.82	1	0
B-10	49	M	138	×	4.95	1	0
B-11	42	F	120	○	5.54	1	0
B-12	55	M	95	×	6.25	1	0
B-13	53	M	142	×	6.73	1	0
B-14	55	F	185	×	6.76	1	0
B-15	57	M	258	×	4.85	1	0
B-16	53	F	175	○	6.33	2	0
B-17	49	M	241	×	5.81	2	0
B-18	44	M	183	×	6.32	2	0
B-19	59	F	167	×	5.89	2	0
B-20	54	M	171	○	6.14	2	0
B-21	52	F	217	×	6.23	2	0
B-22	55	M	222	×	5.88	2	0
B-23	63	F	235	×	6.32	2	0
B-24	47	F	161	×	6.47	2	0
B-25	51	M	96	○	5.31	2	0
B-26	54	F	80	×	5.85	2	0
B-27	55	F	192	○	6.42	3	0
B-28	58	M	234	×	5.93	3	0
B-29	48	M	341	×	4.98	3	0

ALT: Alanine aminotransferase; ISDR: Interferon sensitivity determining region. [1]○ indicates history of transfusion and × indicates no history of transfusion. [2]The unit of HCV RNA titer before treatment (log copies/mL). [3]ISDR type: 1 (wild type, no amino acid substitution), 2 (intermediate type, 1-3 amino acid substitutions), 3 (mutant type, ⩾ 4 amino acid substitutions).

patients. Among them, 21 patients completely responded to the 6-mo combination therapy, while 29 patients showed no response. Group A was designated as the complete response group and group B as the no-response group. The characteristics of each patient prior to combination therapy are shown in Tables 2 and 3.

HCV RNA quantitation

As shown in Tables 2 and 3, the HCV RNA titer had a wide distribution. In the complete response group the HCV RNA titer was between $10^{3.27}$-$10^{6.12}$ copies per mL and $10^{4.85}$-$10^{7.11}$ copies per mL, respectively, in the no-response group. The average RNA titer of the response and no-response groups was 4.62 ± 0.80 and 5.59 ± 0.61, respectively. These values were statistically significant ($P < 0.05$, Table 4).

ISDR and PePHD amino acid sequences

The ISDR and PePHD amino acid sequences and the HCV genotype 1b prototype sequence (HCV-J) are shown in Figure 1. The complete response group had 1-10 amino acid substitutions while the no-response group had 1-8 amino acid substitutions in the ISDR (Figure 1A and B). The PePHD region had 1-2 amino acid substitutions in several cases of complete response group and no amino acid substitutions in the no-response group (Figure 2).

A

2209								2248
	PSLKA	TCTTH	HDSPD	ADLIE	ANLLW	RQEMG	GNITR	VESEN
1	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
2	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
3	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
4	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
5	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
6	- - - R -	- - - - -	- - N - -	P - - - -	- - - - -	- - - - -	- - - - -	- - - - -
7	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
8	- - - - -	- - - - -	- - - - -	V - - - -	- - - - -	- - - - -	- - - - -	- - - - -
9	- - - - -	- - - - -	- - - - -	P - - - -	- - - - -	- - - - -	- - - - -	- - - - -
10	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
11	- - - - -	- - - - C	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
12	- - - - -	- - - - R	- - - - -	V - - - -	- - - - -	- - - - -	- - - - -	- - - - -
13	L - - - -	- - - - -	- - - - -	I - - - D	- - - - -	- - - - -	- - - - -	- - - - -
14	- - - - -	- - - - -	- - - - -	L - - - -	- - - - -	- - - - -	- - - - -	- - - - -
15	V - - - -	A Y I - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
16	- - S - T	- Y I - -	R G - - -	- - - - -	- - - - -	- - - - -	- D - - -	- - - - -
17	L - - - -	A - - - N	- - - - -	V - - - -	- - - - -	- - - - -	- S - - -	- - - - -
18	V - - - -	A - - - -	- - - - -	- - - - -	- - - - -	- - - K -	- T - - -	- - - - K
19	L - - - -	- - R R -	- - - - -	- - D - -	- - - - -	W - - K -	- - - - -	- - - - -
20	L - - - -	- - - R -	N - - V -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
21	O - S - T	- Y I - Q	Y - G - -	V - - - -	- - - - -	- - - - -	- D - - -	- - - - -

B

2209								2248
	PSLKA	TCTTH	HDSPD	ADLIE	ANLLW	RQEMG	GNITR	VESEN
1	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
2	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
3	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
4	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
5	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
6	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
7	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
8	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
9	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
10	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
11	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
12	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
13	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
14	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
15	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
16	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
17	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
18	- - - - -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
19	- - - - -	- - - - C	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
20	- - M - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
21	- - - - -	- - - - C	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
22	- - - - -	- - - - R	- V - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
23	- - - R -	- - - - R	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
24	L - - - -	- - - P -	- - - - -	- - - - -	- - - - -	W - - - -	- - - - -	- - - - -
25	- - - - -	- - - - R	- V - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
26	- - - - -	- - - - -	- - - - -	P - - - -	- - - - -	- - - - -	- - - - -	- - - - -
27	L - - - -	A - - A R	- G F I -	- - - - -	- - - - -	- - - - -	- E - - -	- - - - -
28	- - - - -	- G - - -	- - - - -	- - - - D	- - - - -	P - - - -	- D - - -	- - - - -
29	L - - - -	- - R R -	- - - - -	V - - - -	- - - - -	- - - - -	- - - - -	- - - - -

Figure 1 ISDR sequences (2209-2248) of HCV in complete response group (**A**) and in no-response group (**B**). Dashes indicate the amino acid residues identical to the sequences on the top in each panel.

Response to combination therapy in different groups

As shown in Table 4, patients were younger in the complete response group than in the no-response group. The HCV RNA titer was also significantly different. No differences were found in gender or ALT levels. We classified ISDR sequences into three groups based on the number of amino acid mutations, as previously described[23]. These three groups were analyzed by an odds ratio to define the responsiveness to combination therapy. The ISDR group responses to combination therapy are shown in Table 5. Intermediate (one to three amino acid changes) and mutant (four or more amino acid changes) ISDRs showed an increased responsiveness to the combination therapy. The odds ratio was 2.46 and 7.00, respectively, assuming the wild type had 1.00. The age, PePHD mutations, and HCV RNA titer were significantly different between the two groups (Table 4). Considering these factors, ISDR type might be a better predictive factor for combination therapy responsiveness. After adjusting for age and HCV RNA titer, the odds ratio for intermediate and mutant ISDRs was 3.57

	Complete response group			No-response group		
	RS	ELSPL	LLSTT	RS	ELSPL	LLSTT
1	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
2	- A	- - - - -	- - - - -	- -	- - - - -	- - - - -
3	- Q	- - - - -	- - - - -	- -	- - - - -	- - - - -
4	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
5	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
6	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
7	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
8	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
9	- A	- - - - -	- - - - -	- -	- - - - -	- - - - -
10	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
11	- E	- - - - -	- - - - -	- -	- - - - -	- - - - -
12	- Q	- - - - -	- - - - -	- -	- - - - -	- - - - -
13	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
14	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
15	- -	A- - - -	- - - - -	- -	- - - - -	- - - - -
16	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
17	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
18	- -	Q- - - -	- - - - -	- -	- - - - -	- - - - -
19	- A	Q- - - -	- - - - -	- -	- - - - -	- - - - -
20	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
21	- -	- - - - -	- - - - -	- -	- - - - -	- - - - -
22				- -	- - - - -	- - - - -
23				- -	- - - - -	- - - - -
24				- -	- - - - -	- - - - -
25				- -	- - - - -	- - - - -
26				- -	- - - - -	- - - - -
27				- -	- - - - -	- - - - -
28				- -	- - - - -	- - - - -
29				- -	- - - - -	- - - - -

Figure 2 PePHD sequences (659-670) of HCV in complete response group (n = 21) and no-response group (n = 29). Dashes indicate the amino acid residues identical to the sequences on the top in each panel.

Table 4 Statistical analysis of age, ALT and HCV RNA titer before treatment

Group	Male:Female	Age (yr)	ALT	HCV RNA titer before treatment
A	13:08	49.3 ± 5.0[a]	185.2 ± 89.1	4.62 ± 0.80[b]
B	16:13	52.8 ± 5.7[a]	186.5 ± 61.7	5.95 ± 0.61[b]

ALT: Alanine aminotransferase. The data of age, ALT and HCV RNA titer before treatment are shown in mean ± SD. A: Complete response group. B: No-response group. [a]P = 0.032 between A and B. [b]P = 0.001 between A and B.

Table 5 Logistic regression analysis of ISDR

ISDR type[1]	Crude OR	95% CI	Adjusted OR[2]	95% CI[2]
Wild	1.00		1.00	
Intermediate	2.46	0.64-9.39	3.57	0.66-19.36
Mutant	7.00	1.29-37.91	9.67	1.16-80.65

[1]ISDR wild type had no mutation. Intermediate type had 1-3 mutations and mutant type had more than 4 mutations. [2]After adjustment for age and HCV RNA titer before treatment. OR: Odds ratio; 95% CI: 95% confidence interval.

and 9.67, respectively. According to these results, patients with mutant ISDR strains would likely respond better to combination therapy than those with wild type ISDR strains.

DISCUSSION

Identifying host and viral factors can predict the response of HCV-infected patients to IFN-α and ribavirin combination therapy. Studies showed that factors such as the HCV genotype 1b and viral load are associated with resistance of HCV-infected patients to INF therapy[37,38]. It was reported that resistance of HCV genotype 1b-infected patients to INF therapy is influenced by a region of the NS5A viral phenotype[23,39]. Mutations in this region, known as ISDR, are beneficial for patients receiving IFN therapy, whereas the wild type virus is resistant to IFN treatment[23,39]. Other studies fail to confirm the association between ISDR genotypes and IFN responsiveness[26,27,40-44], thus it remains a controversial issue[45,46].

Combined IFN and ribavirin therapy has replaced IFN monotherapy for HCV-infected patients about 10 years before. In the present study, to identify the predictive factors for effective combination therapy, we investigated the relationship between the response to combination therapy and a variety of factors. Only patients with HCV genotype 1b were studied because this genotype is known to be more resistant to interferon treatment than the other genotypes and is the most prevalent genotype in Korea[15,16].

In this study, age, PePHD mutations, HCV RNA titer, and ISDR subtype were found to be the predictive factors for combined IFN-α and ribavirin therapy for HCV genotype 1b infection. On the other hand, gender and ALT level were not associated with the combination therapy efficacy. These results are consistent with many previous studies, but contrary to others[26,27,31]. Such a difference indicates that these factors are not always accurate predictors for IFN response. This effect may be due to the pleiotropic nature of IFN activity, in addition to other cellular and viral

genes that also modulate the effectiveness of INF therapy for chronic hepatitis C. This is the first study to determine the factors that predict the effectiveness of combination therapy in Korean patients. Therefore, this study may reflect the Korean genetic characteristics.

HCV seems to have a defense strategy against the host cellular responses induced by IFN[47]. The E2 protein appears to play a major role as a potential immune response target, and may interfere with cellular effectors induced by IFN[48]. Information about the clinical implications of E2 containing PePHD, is still limited. Analysis of a small series of HCV genotype 1-infected patients showed that amino acid sequence variability in the PePHD region was similar in responders and no-responders, indicating that the PePHD region is very stable over time[49-51]. In our study, a sequence analysis of the PePHD region in 50 patients found mutations in eight cases, all in the complete response group, suggesting that mutations in the PePHD region are associated with the response to combination therapy. In other studies, a few cases showed some PePHD mutations in the no-response group, though more mutations appeared in the complete response group[31,52]. Therefore, further study is needed to determine why mutations only occur in the complete response group of HCV-infected patients in Korea.

Some studies showed that the association of ISDR mutation rate with treatment response, but the other studies did not[31,45]. One of the Korean studies reported that the effect of INF monotherapy is not associated with the ISDR mutation rate[53]. It is not sure, but the different result may be due to the treatment methods and the sample size.

In conclusion, response of HCV genotype 1b-infected patients to combination therapy is influenced, at least in part, by HCV RNA titer, age, PePHD mutations, and ISDR subtype, but not by gender and ALT level. After adjusting for age and HCV RNA titer, ISDR subtype may be the most potent predictive factor for combination therapy efficacy in Korean chronic hepatitis patients with HCV genotype 1b.

COMMENTS

Background

The effectiveness of IFN-α and ribavirin combination therapy in Hepatitis C virus (HCV) infected patients is not satisfactory, especially in patients with HCV genotype 1b. IFN is also inconvenient to use, costly, and has a variety of possible complications. Therefore, it is necessary to determine the predictive factors for IFN therapy.

Research frontiers

It is very important to know the pathogenesis of HCV-infected patients to block the progression of hepatocellular carcinoma.

Innovations and breakthroughs

This is the first study to determine the factors that predict the effectiveness of IFN-α and ribavirin combination therapy in Korean patients. In this study we used a large number of samples to determine the ISDR subtype, HCV RNA titer, age, and PePHD mutations.

Applications

The predictive factors for IFN-α and ribavirin combination therapy in patients with HCV genotype 1b can be used to select its candidates.

Peer review

This article is of theoretical and practical importance. The results show that HCV RNA ISDR type may be an important factor for predicting the efficacy of IFN-α and ribavirin combination therapy in Korean patients.

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RAPID COMMUNICATION

Effect and mechanism of β-L-D4A on DNA polymerase α

Yan Li, Ju-Sheng Lin, Ying-Hui Zhang, Xiao-Yan Wang, Ying Chang, Xing-Xing He

Yan Li, Ju-Sheng Lin, Ying-Hui Zhang, Xiao-Yan Wang, Ying Chang, Xing-Xing He, Institute of Liver Diseases, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Street, Wuhan 430030, Hubei Province, China
Supported by National Natural Science Foundation of China, No. 30330680
Correspondence to: Dr. Ju-Sheng Lin, Professor, Institute of Liver Diseases, Tongji Medical College, 1095 Jiefang Street, Wuhan 430030, Hubei Province, China. jslin@tjh.tjmu.edu.cn
Telephone: + 86-27-83663661 Fax: +86-27-83663661
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Abstract

AIM: To investigate the safety of β-L-D4A on DNA polymerase α.

METHODS: Ion exchange chromatography was used to separate DNA polymerase α from crude extract of human Hela cells. Detailed kinetic parameters were determined for β-L-D4A against DNA polymerase α.

RESULTS: DNA polymerase α was purified with 4% yield and 31000 units/mg specific activity. The Michaelis constant (Km = 3.22 μmol/L), 50% inhibition concentration (IC50 = 178.49 μmol/L) and inhibition constant (Ki = 126 μmol/L) of β-L-D4A were determined by kinetic analysis.

CONCLUSION: β-L-D4A is a more safe nucleoside for hepatitis B virus (HBV) infection with a lower host toxicity.

Key words: Nucleoside; β-L-D4A; DNA polymerase α; Kinetic study; Side effect; Hepatitis B virus

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INTRODUCTION

Infection with hepatitis B virus (HBV) is a global medical problem[1]. With the support of National Natural Science Foundation of China[2], we have found a novel nucleoside analog (β-L-D4A), a potent and selective inhibitor of HBV replication, which is stronger than lamivudine (3TC)[3,4]. Because the nucleoside and nucleoside analog could serve as substrates for normal DNA polymerases, resulting in inhibition of cellular DNA synthesis, we decided to study the side effect of β-L-D4A on DNA polymerases[5].

It has been found that many kinds of DNA polymerase have a crucial role in DNA replication, among which, the high molecular weight class of eukaryotic DNA polymerase α (EC2.7.7.7), first identified in calf thymus extract and found to be ubiquitous among eukaryotes, constitutes the predominant polymerase species in actively dividing cells[6,7]. So ion exchange chromatography was used to separate DNA polymerase α from crude extract of human Hela cells, which could be used as a substrate to evaluate the safety of nucleoside-β-L-D4A.

MATERIALS AND METHODS

Reagents

β-L-D4 triphosphate was chemically synthesized in our institute with the help of Pharmaceutic College of Wuhan University and identified by infrared mass spectra, nuclear-magnetic resonance. 3TC 5' triphosphate was provided by Professor Cheng YC (School of Medicine, Yale University, New Haven, CT). Hela S3 cells were purchased from Institute of Immunity, Tongji Medical College, DE52 and P11 cellulose resin were from Whatman while HTP was from Bio-Rad. DNA-cellulose and activator calf thymus DNA were obtained from SIGMA. α-^{32}P-dTTP was from Amersham. All other tissue culture reagents were from Gibco.

All operations were performed at 0℃-4℃. Unless otherwise noted, all buffers contained 1 mmol/L β-mercaptoethanol and 1 mmol/L EDTA.

Preparation of cytoplasmic crude extract

Frozen cells (approximately 80 g) were suspended in 300 mL of 2 mmol/L $MgCl_2$, 1 mmol/L p-toluenesulfonyl fluoride, 10 mL/L isopropyl alconol, and 5 mmol/L KPO_4 (pH7.5), and broken in a Dounce homogenizer after 20 min at 0℃. Nuclei were spun down at 800 r/min for 10 min, and mitochondria were pelleted at 13000 r/min for 20 min. The mitochondrial pellet was washed with 80 mL extraction buffer and re-sedimentated with the two supernatants combined (a). Fraction a was dialyzed for 8-12 h against 10 volumes twice with 0.2 mol/L sodium acetate (pH5.5) each time, after which a flocculent precipitate was removed by centrifugation at 13000 r/min for 20 min (b). Fraction b was over-layered with 117 mL/L sucrose in 0.2 mol/L KPO_4 (pH8.2) and spun for 90 min at 40000 r/min in a Beckman rotor. The supernatant was saved (c).

First DEAE-cellulose chromatography

Fraction c was diluted to a final concentration of 0.1 mol/L KPO_4 at 1:1 with 1 mmol/L β-mercaptoethanol, 1 mmol/L EDTA and loaded onto a DEAE-cellulose column (5 cm × 20 cm) equilibrated with the same buffer. The column was washed with 3 volumes of equilibration buffer, and the adsorbed activity was then eluted in a single step with 0.2 mol/L KPO_4 (pH8.2). All the active fractions from the step were pooled (d).

Second DEAE-cellulose chromatography

Fraction d was diluted to a final concentration of 0.1 mol/L KPO_4 with 1 mmol/L β-mercaptoethanol, 1 mmol/L EDTA and loaded onto a second DEAE-cellulose column (2.5 cm × 25 cm). The column was washed with 3 volumes of equilibration buffer and then developed with a 5-6 column volume gradient from 0.1 mol/L to 0.2 mol/L KPO_4 (pH8.2).

Phosphocellulose chromatography

The peak fractions of protein obtained from DE52 were pooled and dialyzed against 300 g/L sucrose, 200 mL/L ethylene glycol, 0.1 mol/L KPO_4 (pH7.2) (e). Fraction e was loaded onto a phosphocellulose column (2.5 cm × 20 cm). The column was developed with a 6-volume gradient from 0.1 mol/L to 0.3 mol/L KPO_4 (pH7.2).

Hydroxylapatite chromatography

The peak fractions of DNA polymerase α from P11 were pooled and dialyzed against 300 g/L sucrose, 0.05 mol/L KPO_4 (pH 6.8) (f). Fraction f was diluted at 1:1 with 1.0 mol/L KCl, 4 mL/L Triton X-100 and loaded onto a hydroxylapatite column (1 cm × 13 cm). The column was eluted with a 15-volume gradient from 0.025 mol/L to 0.2 mol/L KPO_4 (pH7.2).

DNA-cellulose chromatography

The peak fractions were pooled and concentrated by dialysis against 300 g/L sucrose, 0.1 mol/L KPO_4 (pH7.5) (g). Fraction g was diluted to a final concentration of 0.02 mol/L KPO_4 (pH7.5) at 1:4 with 1 mmol/L β-mercaptoethanol, 1 mmol/L EDTA, and loaded onto a DNA cellulose column (1 cm × 10 cm). The column was developed with a 20-volume gradient from 0.02 mol/L to 0.2 mol/L KPO_4 (pH7.5).

Protein concentration

Bradford method was used to measure the protein concentration, which was operated by directions of the reagent box.

Assay of DNA polymerase α

The final volume of reaction mixture was 50 μL containing 2 μL DNA polymerase α, 50 μmol/L d CTP, 50 μmol/L d GTP, 50 μmol/L d ATP, 50 μmol/L α-^{32}Pd TTP (100 cpm/pmol), 100 μg/mL activated calf thymus DNA, 50 mmol/L Tris-HCl (pH7.5), 0.5 mmol/L $MnCl_2$, 100 mmol/L KCl and 2.5 mmol/L DTT. The mixture was Incubated at 37℃ for 15 min, and spotted onto DE81 filter paper. The paper was washed three times with 5% Na_2HPO_4 (10 min each time), twice with water (5 min each time), dried and assayed for the acid-insoluble radioactivity.

Sodium dodecyl sulfatd polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to detect the purity of DNA polymerase α.

Western blot

Western blot was performed to confirm the protein bands.

Detailed kinetic parameters were determined using DNA polymerase α which was prepared, pooled and stored at -80℃ until use in experiments. Potential inhibitors at various concentrations were added to 5 μL reaction mixture. Control samples (without inhibitor) included 5 μL solution in PBS, but no inhibitors. DNA polymerase α activity was measured.

Michaelis constant (Km)

To find the inhibition type, α-^{32}P-dTTP(100 cpm/pmol) was performed at the concentration of 1, 3, 5, 7, 10 μmol/L while inhibitors were added at the concentration of 10 μmol/L of each compound. DNA polymerase α was determined. The data were analyzed with GraphPad Prism 4 Demo, which could finish Line weaver-Burk Plot and L-B linear secondary regression with the enzyme kinetics template.

Fifty percent inhibition concentration (IC50)

To determine the concentration required to inhibit DNA polymerase α activity by IC50, initial experiments were designed with the gradient (1 μmol/L-400 μmol/L) of each compound. The concentration of α-^{32}P-dTTP (100 cpm/pmol) was 3 μmol/L. All experiments were performed twice. Inhibition rate = (1 - count of drug class/blank control) × 100%. Half logarithm plot was used to process the data. DNA polymerase α activity in the control sample was set at 100%.

Inhibition constant (Ki)

To find the inhibition constant (Ki), each compound was studied at five different inhibitor concentrations (10, 40, 100, 160, 200 μmol/L), and at various substrate concentrations (2, 5 μmol/L) in duplicate. Dixon plot was used to deal with the data.

RESULTS

DEAE-cellulose chromatography

The first DE52 chromatography was to remove nucleic acid. The curve of A280 showed a broad peak including 74 tubes from the 16th tube (0.115 mol/L) to the 90th tube (0.185 mol/L) in the second DE52 chromatography (Figure 1A). The protein in 74 tubes was collected and the ingredient was confirmed by assay of DNA polymerase α activity. The total protein was 0.36 mg measured by Bradford method.

Phosphocellulose chromatography

Protein determined by A280 was eluted sharply as a

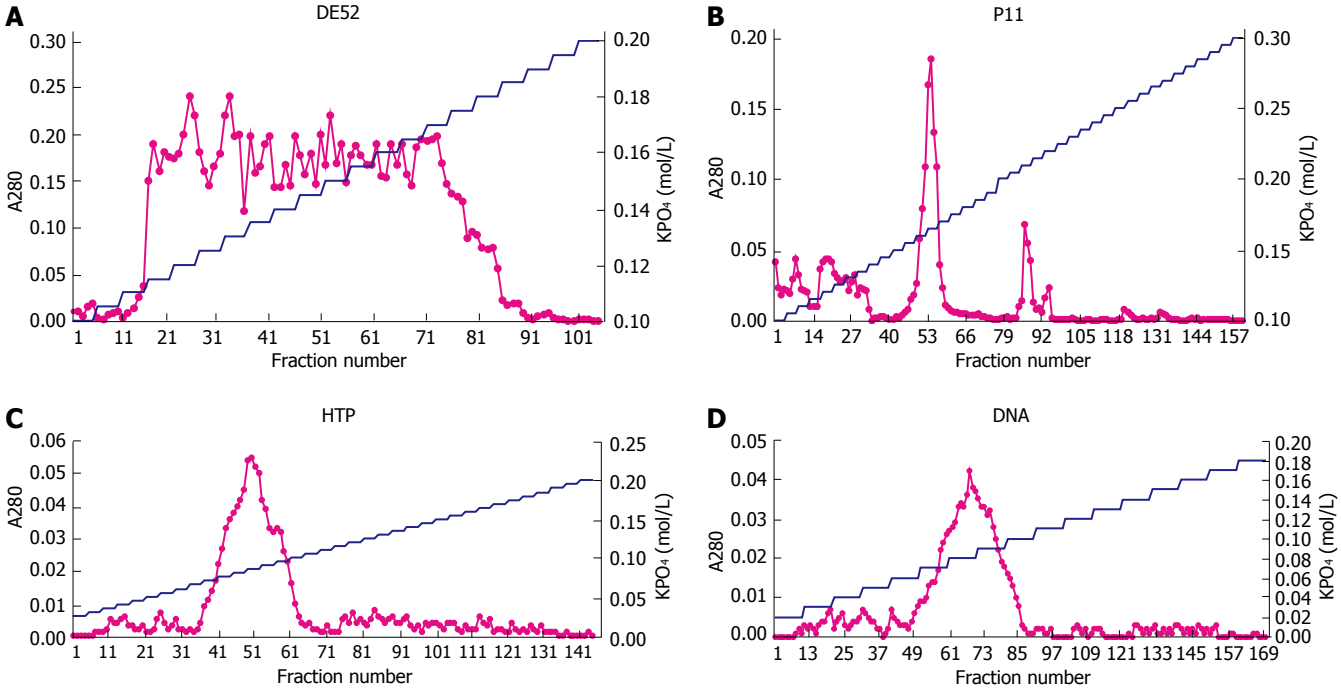

Figure 1 A280 and gradient concentration of KPO_4 in each chromatography. The curve with spots is for A280, the other is for KPO_4. **A**: A broad peak of protein from the 16th tube (0.115 mol/L) to the 90th tube (0.185 mol/L), clearly observed in the second DE52 chromatography; **B**: Phosphocellulose chromatography displaying a major peak of protein identified at 0.165 mol/L KPO_4 and a minor trailing shoulder at 0.21 mol/L; **C**: Hydroxylapatite chromatography showing a single sharp peak of protein at 0.085 mol/L KPO_4; **D**: DNA-cellulose chromatography revealing a single sharp peak at 0.085 mol/L KPO_4.

Table 1 Protein, activity, specific activity, purification times and yield of each step

Step	Protein/ mg	Activity/ units	Specific activity/ (units/mg)	Purification times	Yield(%)
Crude extract	9.000	800	89		
First DEAE	0.880	480	545	6	60
Second DEAE	0.360	260	722	8	33
P11	0.039	240	6154	69	30
HTP	0.008	120	15000	169	15
DNA	0.002	32	31000	348	4

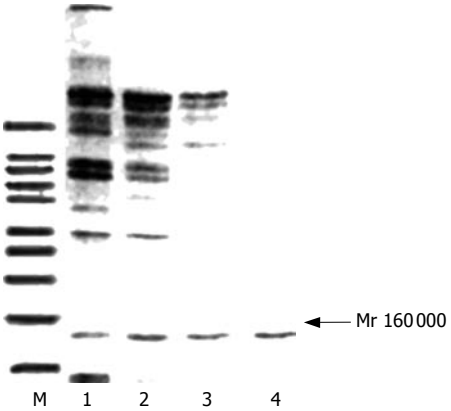

Figure 2 SDS-PAGE displaying decreased bands after purification steps-DE52 (lane 1), P11 (lane 2), HTP (lane 3) and DNA (lane4), and M (marker), While DNA-cellulose chromatography demonstrating only one band (M_r 160 000).

major peak at 0.165 mol/L, with a minor trailing shoulder occasionally present at 0.21 mol/L (Figure 1B). Fractions from 0.15 mol/L to 0.18 mol/L KPO_4 were pooled because the minor peak was not the protein as expected. The total protein was 0.039 mg.

Figure 3 Western blot of the fractions of DNA-cellulose chromatography. When mouse anti-human DNA polymerase α monocloned antibody was used as the primary antibody, the fraction was found to be DNA polymerase α.

Hydroxylapatite chromatography

Fractions from the 37th to the 64th tube were collected because a single sharp peak appeared at 0.085 mol/L KPO_4 (Figure 1C). The characterization was certified by its activity and the protein was 0.008 mg.

DNA-cellulose chromatography

Protein was eluted in a single sharp peak at 0.085 mol/L KPO_4 (Figure 1D). As the activity was measured, the region from 0.07 mol/L to 0.10 mol/L KPO_4 was pooled and concentrated by dialysis against 300 g/L sucrose, 0.1 mol/L KPO_4 (pH7.5).

Analysis of protein and activity

When the specific activity became higher, the total protein, total activity and yield became lower in each stage, illustrating that DNA polymerase α was purified step by step while the other components were removed (Table 1).

SDS-PAGE and Western blot

SDS-PAGE showed that the purification of protein was effective because the bands decreased with the step of purification and finally there was only one band (Figure 2). Western blot revealed that the band was DNA polymerase α (Figure 3).

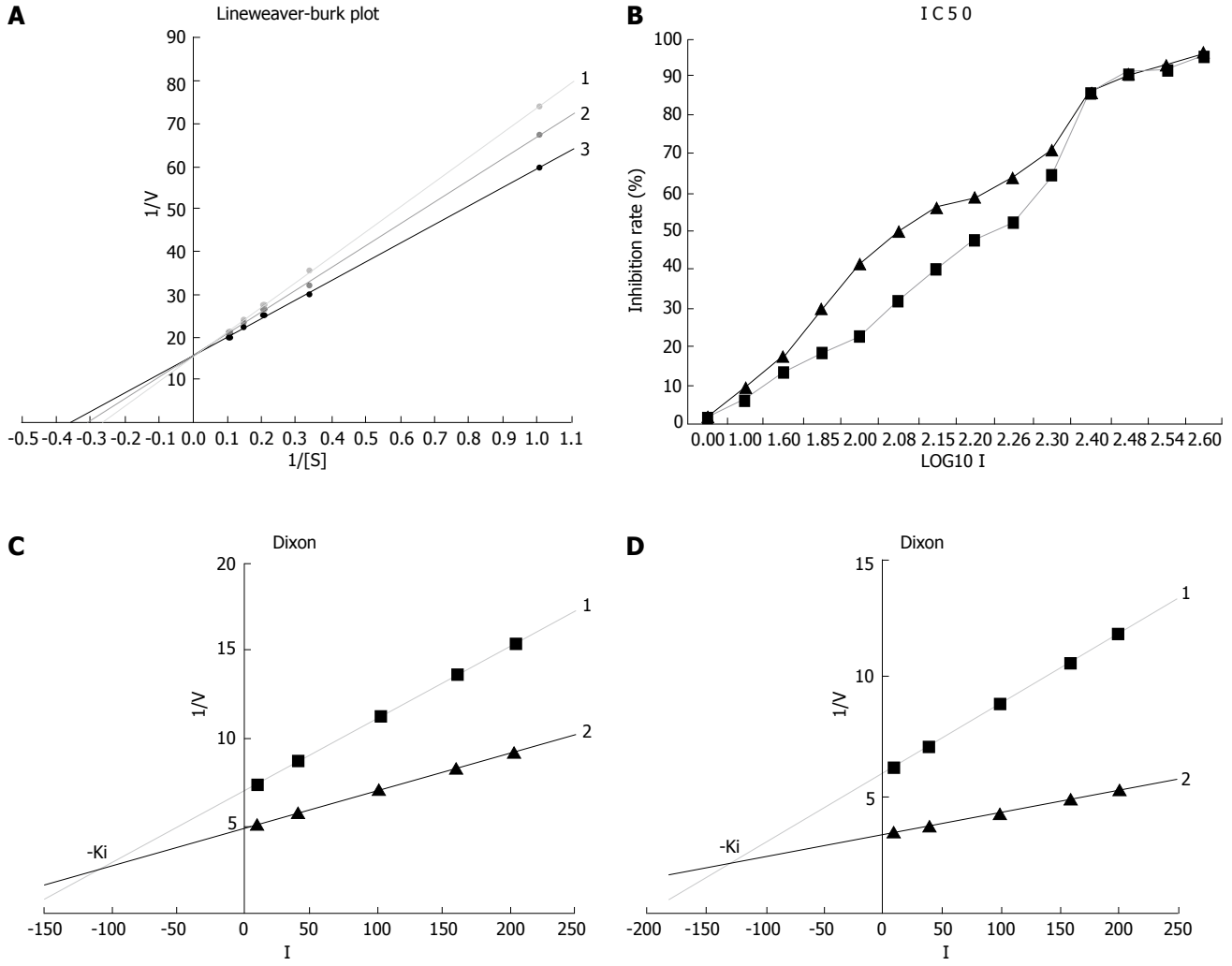

Figure 4 Determination of detailed kinetic parameters Km (**A**), IC50 (**B**), and Ki (**C** for 3TC, **D** for β-L-D4A).

Michaelis constant (Km)

The inhibition mechanism was observed by visual inspection of graphical plots after data linearization (Line weaver-Burk plot) using the GraphPad Prism 4 Demo software. The L-B linear regression equations in Figure 4A showed 3TC5'-triphosphate, y = 15.922 + 57.978x (r^2 = 0.9999), Km = 3.64 μmol/L; β-L-D4A, y = 15.879 + 51.163x (r^2 = 0.9993), Km = 3.22 μmol/L; blank without inhibitor, y = 15.860 + 43.493x (r^2 = 0.9995), Km = 2.74 μmol/L.

Fifty percent inhibition concentration (IC50)

IC50 was calculated using the half logarithm plot, demonstrating that β-L-D4A (178.49 μmol/L) was 1.5 fold more potent than 3TC (122.41 μmol/L) (Figure 4B).

Inhibition constant (Ki)

The Ki was also estimated using the GraphPad Prism 4 Demo software. The two linear regression equations in Figure 4C were 3TC, y = 0.0408x + 6.9594 (r^2 = 0.9998) and y = 0.0212x + 4.8957 (r^2 = 0.9991) while the equations in Figure 4D were β-L-D4A, y = 0.0297x + 6.0389 (r^2 = 0.9965) and y = 0.0095x + 3.4904 (r^2 = 0.9996). The Ki of 3TC was 105 μmol/L which was lower than β-L-D4A (126 μmol/L).

DISCUSSION

HBV infection, associated with the risk of developing liver cirrhosis and hepatocellular carcinoma, is a worldwide public health problem. Two billion people worldwide show evidence of having been infected with HBV, and more than 350 million of them are chronically infected. Persons with chronic hepatitis B are at a high risk of developing hepatic cirrhosis and primary hepatocellular carcinoma, leading to 500000 to 1.2 million deaths annually worldwide. Antiviral chemotherapy remains the only choice of treatment for controlling HBV infection in these individuals, for whom available HBV vaccines provide no benefit. At present, approved therapeutics for chronic HBV infection are α-interferon or nucleoside and nucleoside analogs[8]. Drawbacks of treatment with α interferon include a low sustained response rate, undesirable side effects, the need for parenteral administration, and high cost[9,10]. Treatment with nucleosides such as 3TC is less costly and more convenient[11,12]. The fundamental concern is that while initial treatment of patients with 3TC results in a rapid decrease in HBV DNA blood levels, its efficacy is severely compromised in most patients by the development of antiviral resistance after prolonged therapy[13,14]. Although the use of nucleoside and nucleoside analogs as anti-

hepadnaviral agents is similarly disappointing, prospects for their future use are bright, as several of recently developed analogs have been found to be potent and can be used as selective inhibitors of HBV replication. In our previous work, the novel nucleoside (β-L-D4A) was synthesized and its inhibitiory actions against HBV were studied and found to be much better than 3TC[15,16]. The inhibition of HBV replication by nucleoside analogs results from the recognition of nucleoside analog triphosphates (TPs) by the RNA-dependent DNA polymerase of HBV (HBV Pol). The triphosphate derivatives could serve as substrates for human DNA polymerases, inhibiting cellular DNA synthesis. For the systemic evaluation of the safety of the new drug, kinetic analysis of DNA polymerases must be performed.

Of the multiple DNA polymerases occurring in eukaryotes, only DNA polymerase α is able to initiate the synthesis of new strands[6], because it can initiate the replication of DNA by cooperating with RNA polymerase[17-19].

In the present study, DNA polymerase α was purified step by step because the total protein and activity decreased while the specific activity raised and the number of protein bands was cut down till only one band was left in SDS-PAGE (Mr 160 000). Western blot and the specific activity (31 000 units/mg) confirmed that the scheme was effective. The enzyme obtained lays a foundation for the next research.

Nucleoside analogs are chemically synthesized drugs that are able to mimic natural nucleosides[20,21]. They exert their antiviral effect, after anabolism to the triphosphate form, by acting as alternate substrates for the virally encoded reverse transcriptase[22,23]. Incorporation of the nucleoside analog monophosphate into the viral DNA, results in premature termination of viral DNA synthesis. Nucleoside analogs competitively inhibit DNA-dependent reverse transcriptase activity of the viral polymerase[24,25]. As DNA polymerase α has the similar substrate and mechanism to HBV viral polymerase, the drugs could serve as substrates for human DNA polymerase α, inhibiting cellular DNA synthesis[26,27]. Because DNA polymerase α exhibits neither exonuclease nor endonuclease activity, mistake cannot be repaired[28,29]. To determine the safety of nucleosides on cells in the present study, DNA polymerase α was purified and kinetic analysis was performed under the identical conditions of ionic strength, pH, divalent metal ion concentration, and DNA substrate.

The Km increased and Wmax unchanged (Figure 4A), confirming that both of β-L-D4A and 3TC are the competitive inhibitors of DNA polymerase α. In our study, the drugs inhibited DNA polymerase α activity by acting as competitive alternate substrates. 3TC was more effective than β-L-D4A as inhibitors of DNA polymerase α because IC50 of β-L-D4A was 1.5 fold more potent than 3TC and the Ki of 3TC was 105 μmol/L, much lower than β-L-D4A (126 μmol/L), demonstrating that β-L-D4A is significantly more safe than 3TC.

In conclusion, β-L-D4A is a safe drug for HBV infection because it is endowed with lower host toxicity in comparison to 3TC. Furthermore, combined therapy of β-L-D4A and lamivudine for HBV infection can be explored.

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COMMENTS

Background

Since hepatitis B virus (HBV) replication involves a virally encoded reverse transcriptase (RT), a number of L-configuration nucleoside analogs with an inhibitory effect on RT have emerged as potent antiviral agents against HBV infection. However, most anti-HBV nucleoside analogs tested to date have only transient and limited effects on a small number of HBV-infected individuals with moderate to severe side effects. In light of the fact, development of novel antiviral agents is an extremely important undertaking.

Research frontiers

In recent years, a considerable interest has been focused on the use of 2', 3'-dideoxynucleosides (DDNs) in the treatment of chronic HBV infection. β-L-D4A, a novel L-nucleoside, can effectively block the production of HBV in 2.2.15 cells *in vitro*, but no information is available on the safety evaluation of this new compound.

Innovations and breakthroughs

In this research, kinetic analysis of DNA polymerase α cytotoxicity, was investigated to evaluate the safety of this new compound.

Applications

β-L-D4A is a safe drug for HBV infection because it is endowed with a low host toxicity.

Terminology

β-L-D4A, [5-(6-amino-9H-purin-9-yl)-2,5-dihydrofuran-2-yl] methanol, is a novel L-nucleoside.

Peer review

This is an interesting paper describing the effect of β-L-D4A (a novel nucleoside analog) on DNA polymerase α. The authors separated DNA polymerase α from crude extract of human Hela cells and studied its enzyme kinetics, showing that the enzyme is less inhibited than lamivudine, thus β-L-D4A may be an effective nucleoside for HBV infection with a lower host toxicity.

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RAPID COMMUNICATION

Effects of endogenous nitric oxide induced by 5-fluorouracil and L-Arg on liver carcinoma in nude mice

Xiao-Yan Yin, Jun-Mei Jiang, Ji-Yong Liu, Ju-Ren Zhu

Xiao-Yan Yin, Endoscopy Center, Affilated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China
Jun-Mei Jiang, Ji-Yong Liu, Ju-Ren Zhu, Department of Gastroenterology, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China
Supported by the Grants for the Excellent Youth Scientists of Shandong Provincial Science Commission. No. 2000BB2DBA1
Correspondence to: Dr. Jun-Mei Jiang, Department of Gastroenterology, Shandong Provincial Hospital, Jinan 250021, Shandong Province, China. jiangjunm@hotmail.com
Telephone: +86-532-82911525 Fax: +86-532-82911999
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Abstract

AIM: To study the effects of endogeous nitric oxide induced by 5-fluorouracil (5-FU) and L-arginine (L-Arg) on the human liver carcinoma model in nude mice.

METHODS: The human liver carcinoma model in nude mice was established with BEL-7402 cells and normal saline (NS), 5-FU and 5-FU + L-Arg injected intraperitoneally. The tumor size was measured. The necrotic degree and range were observed under microscope. The apoptosis of cancer cell was detected by turmina deoxynucleotidyl transferanse mediated dUTP nick end labeling (TUNEL) method. Immunohistochemical method was performed to determine the expression of iNOS, P16, BAX. The chemical colorimetry was used to test the activity and nitrate reductase method was adopted to test the concentration of nitric oxide (NO) in the tumor tissue. The BI2000 pathological image analyzer was used to analyze the result of immunohistochemistry.

RESULTS: 5-FU combined with L-Arg could inhibit the tumor growth apparently. In NS, 5-FU and 5-FU+L-Arg groups, the changes of tumor volumes were 257.978 ± 59.0, 172.232 ± 66.0 and 91.523 ± 26.7 mm^3, respectively ($P < 0.05$ 5-FU *vs* 5-FU + L-Arg group; $P < 0.05$ NS *vs* 5-FU + L-Arg group; $P < 0.05$, NS *vs* 5-FU group). The necrotic range and apoptosis index were significantly increased after the drug injection. The necrotic range was biggest in 5-FU + L-Arg group ($\chi^2 = 15.963$, $P < 0.05$). The apoptosis indexes were as follows: NS, 17.4% ± 6.19%; 5-FU, 31.3% ± 12.3%; and 5-FU + L-Arg, 46% ± 15.24% ($P < 0.05$, 5-FU *vs* 5-FU + L-Arg; $P < 0.05$, NS *vs* 5-FU + L-Arg; $P < 0.05$, NS *vs* 5-FU). The expression and activity of iNOS were increased in the tumor tissue. The concentration of NO was also increased. *F* of optical density of iNOS, iNOS activity and NO concentration are 31.693, 21.949, and 33.909, respectively, $P < 0.05$. The concentration of NO was related to the expression of P16 and BAX. The correlation coefficient was 0.764 and 0.554.

CONCLUSION: 5-FU combined with L-Arg can inhibit the growth of tumor in nude mice. The effect may be related to inducing the synthesis and increasing the activity of iNOS. The production of NO is increased, and it can enhance the expression of apoptosis-related gene and antioncogene.

Key words: 5-Fluorouracil; L-Arginine; Animal model; Nitric oxide synthase; Nitric oxide

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INTRODUCTION

Over the past decade or so, it has become evident that free radical gas nitric oxide acts as a novel transcellular messenger molecule in many key physiological and pathological processes. It appears that high levels of nitric oxide (NO) may be cytostatic or cytotoxic for tumor cells[1,2]. NO is a small gaseous molecule generated in a wide variety of cells as a product of the conversion of L-arginine into L-citrulline by the enzyme nitric oxide synthase (NOS). There have been few reports about the antitumor effects of endogenous nitric oxide *in vivo*[3,4]. In this study, we observed the inductive effects of 5-fluorouracil (5-FU) and L-arginine (L-Arg) on the endogenous nitic oxide in the human live carcinoma model in nude mice and proved the antitumor effects of the endogenous nitric oxide in the primary hepatic carcinoma and found effective adjuvant for 5-FU.

MATERIALS AND METHODS

Materials

Male Balb/c nude mice were provided by the animal center of Shandong University. BEL-7402 cells were obtained from Shandong Academy of Medical Sciences, China.

Immunochemical kit and *in situ* cell apoptosis detection kit were purchased from Wuhan Boster Technology Company, China. Nitric oxide synthase and NO detection kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. 5-FU was produced by Shanghai Xudong Haipu Pharmacentical Company. L-Arg was produced by Shanghai Xinyi Jinzhu Pharmacentical Company.

Cell lines and culture conditions

Human primary hepatocellular cancer cell line BEL-7402 was maintained in a humidified, 5% CO_2 atmosphere and cultured in DMEM, supplemented with 10% fetal calf serum and penicillin-streptomycin mixture.

Grouping and animal model

BEL-7402 cells were cultured *in vitro* to the log growth phase, centrifuged and washed with PBS to a concentration of 1×10^7/mL, and 0.2 mL cell solution was subcutaneously xenografted in the neck or the flank of 4-6 wk old male Balb/c nude mice. The mice were housed and maintained in lamina flow cabinets under specific pathogen-free conditions. Ten day after establishment of the model when tumors reached a mean diameter of 0.5 cm, mice were randomly divided into three groups (10 mice per group) and treated as follows: group A was injected intraperitoneally with 0.2 mL normal saline, group B was injected with 5-FU at a dose of 20 mg/kg, and group C was injected with 5-FU at 20 mg/kg and L-Arg at 100 mg/kg. The drugs were injected every other day from d 11 to d 15. The mice were killed at 17d after the experiment finished.

Tumor inhibition rate in vivo

The largest diameter (a) and its vertical diameter (b) of the tumors were measured with caplipers every two days after the tumor appeared. The volume of tumor was equal to $1/2ab^2$. The tumor inhibition rate (TIR) was calculated with the following formula:

TIR = control group (v_1 - v_0) - treatment group (v_1 - v_0)/control group (v_1 - v_0)

(v_0: the volume before injection of drugs, v_1:the volume after injection of drugs).

Histology

The sections were stained with hematoxylin-eosin to evaluate the degree and range of the necrosis. It was divided into 4 degrees according to the percentage of the necrotic area: ≤ 25% (+), 25%-50% (++), 50%-75% (+++), > 75% (++++)[5].

TUNEL

TUNEL method was used to detect the apoptosis of tumor cells according to the instructions of the manufacturer. Cells with brown or yellow nuclei were assumed as apoptotic cells. The number of apoptotic cells and total cancer cells was counted under light microscope at 400 × magnification in 5 fields of version and the average values were used for the calculation of apoptosis index (AI) according to the formula: AI = (apoptotic cells/total cancer cells) × 100%.

Immunohistochemical staining

Tumor specimens were dissected from mice and fixed in 10% buffered formalin solution overnight. They were then embedded in paraffin and sectioned in 4 μm thicknesses. SABC immunohistochemistry was performed according to the manufacturer's instructions to detect the gene expression of iNOS, P16 and BAX. Briefly, the tissue sections were deparaffinized in xylene at 37℃ for 20 min. Endogenous peroxide was blocked by incubating the slides with 30 mL/L H_2O_2 for 10 min at 37℃. Sections were incubated with primary antibodies of iNOS, P16 and BAX at 4℃ overnight respectively. Staining was visualized with DAB for 10 min at room temperature. Finally, the sections were counterstained for nuclei by hematoxylin solution. The results were analyzed with a BI2000 pathological image analyzer and expressed as optical density.

iNOS activity and NO concentration assay

NOS catalyzed the formation of NO and L-citrulline from L-arginine and molecular oxygen, and NO reacted with a nucleophile to generate color compounds. The absorbance of NOS at 530 nm was calculated and expressed as U/mg protein. One unit of NOS activity was defined as the production of 1 nmol nitric oxide per second per mg tissue protein. Total NOS activity was measured as follows: 10% tissue homogenate (100 μL) was incubated with 200 μL substrate buffer, 10 μL reaction accelerator and 100 μL color development reagent at 37℃ for 15 min after mixing. Then 100 μL clearing reagent and 2 mL stop solution were added, mixed and absorbances were read at 530 nm. For measuring iNOS activity, an inhibitor was added before incubation according to the manufacturer's instructions. Total protein concentration was determined using the Coomassie blue method with bovine serum albumin as standard.

Tumor samples were thawed, weighed and homogenized 1:9 w:v in 0.9% saline. The homogenates were then centrifuged at 1000 r/min for 5 min at 4℃, the supernatant was taken for NO assay and total protein determination.

NO was assayed spectrophotometrically by measuring total nitrate plus nitrite (NO_3^- plus NO_2^-) and the stable end products of NO metabolism. The procedure was performed by the manufacturer's instructions. Results were expressed as μmol/g protein.

Statistical analysis

All data were analyzed with SPSS11.0 statistical software. One-way ANOVA, Bonferroni, Kruskal-Wallis H test and correlated analysis were performed.

RESULTS

Tumor inhibition rate in vivo

5-FU could inhibit the growth of the liver carcinoma in nude mice. When the substrate L-Arg was supplemented, tumor inhibition rate increased significantly (Table 1).

Histology

There was different necrosis in the tumor tissues. The

Table 1 Changes of tumor volume and tumor inhibition rate

	n	Changes of tumor volume (mm^3)	Tumor inhibition rate (%)
A: NS group	10	257.978 ± 59.0[a]	
B: 5-FU group	10	172.232 ± 66.0[c]	33.24
C: 5-FU+L-Arg group	10	91.523 ± 26.7[e]	64.52

[a]$P < 0.05$, group B *vs* C; [c]$P < 0.05$, A *vs* C; [e]$P < 0.05$, A *vs* B.

Table 2 Necrotic range in tumor tissues

	+	+ +	+ + +	+ + + +
A: NS group	6	3	1	
B: 5-FU group		5	3	2
C: 5-FU+L-Arg group		1	4	5

$\chi^2 = 15.963$, $P < 0.05$.

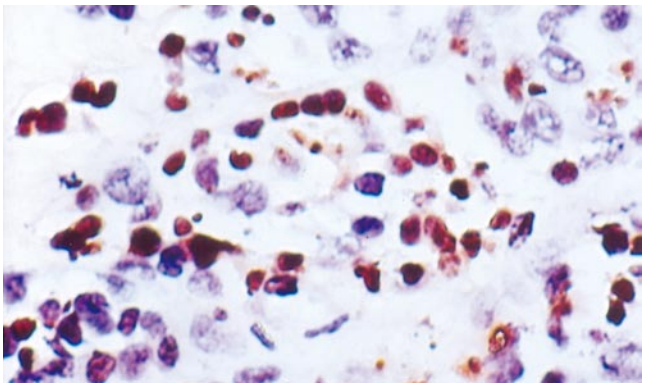

Figure 1 The apoptosis cells in tumor tissues (× 400).

necrotic range enlarged greatly after treatment with 5-FU and L-Arg (Table 2).

Apoptosis index

There were a lot of apoptosis cells in 5-FU and 5-FU+L-Arg groups, while only scattered apoptosis cells in NS group. The apoptosis cells are shown in Figure 1. The apoptosis indexes were as follows: NS group 17.4% ± 6.19%; 5-FU group 31.3% ± 12.3%; and 5-FU+L-Arg group 46% ± 15.24%. [a]$P < 0.05$ group B *vs* group C; [c]$P < 0.05$ A *vs* C; [e]$P < 0.05$ A *vs* B. There was significantdifference between the two groups.

iNOS activity, NO concentration and immunohistochemical results of iNOS

The optical density of iNOS, iNOS activity and NO concentration were increased after treatment with 5-FU. When the substrate L-arg was supplemented, they were increased more significantly (Table 3). The expression of iNOS is shown in Figure 2.

Correlation analysis between concentration of NO and expression of P16, BAX

The expression of P16 and BAX was increased after

Table 3 Optical density of iNOS ,iNOS activity and NO concentration in tumor tissues

	Optical density of iNOS (μmol/L)	Activity of iNOS (U/mg protein)	Concentration of NO
A: NS group	0.4621 ± 0.0115[a]	4.87 ± 2.5[a]	6.58 ± 3.2[a]
B: 5-FU group	0.4783 ± 0.0107[c]	9.83 ± 2.31[c]	17.97 ± 6.16[c]
C: 5-FU+L-Arg group	0.5269 ± 0.0034[e]	15.1 ± 4.91[e]	30.41 ± 8.81[e]

F of optical density of iNOS , iNOS activity and NO concentration are 31.693, 21.949 and 33.909, respectively, [a]$P < 0.05$, B *vs* C; [c]$P < 0.05$, A *vs* C; [e]$P < 0.05$, A *vs* B.

Table 4 The optical density of P16 and BAX in tumor tissues

	Optical density of BAX	Optical density of P16
A: NS group	0.4501 ± 0.0114[a]	0.4565 ± 0.0139[a]
B: 5-FU group	0.4788 ± 0.0068[c]	0.4939 ± 0.041[c]
C: 5-FU+L-Arg group	0.5045 ± 0.0199[e]	0.5451 ± 0.027[e]

F of optical density of BAX andP16 is 23.51 and 16.13. [a]$P < 0.05$, B *vs* C; [c]$P < 0.05$, A *vs* C; [e]$P < 0.05$, A *vs* B.

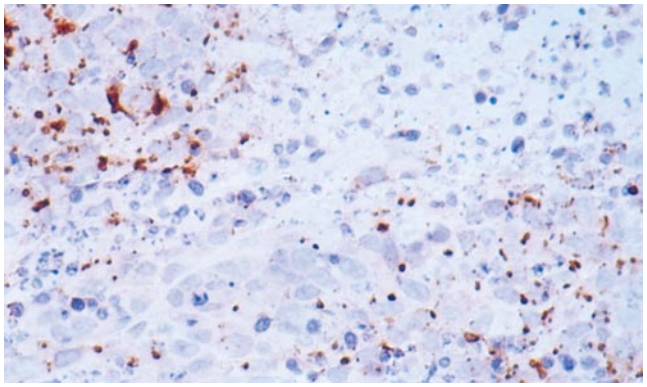

Figure 2 The expression of iNOS in tumor tissues (× 200).

treatment with 5-FU and L-Arg. $P < 0.05$ (Table 4). The result of Pearson analysis showed that the concentration of NO was correlated with the expression of P16 and BAX. The correlation coefficient was 0.764 and 0.554.

DISCUSSION

Hepatocellar carcinoma is one of the most common malignant tumors in China. Surgical resection is the best choice. But many patients could not be treated surgically when diagnosis was made too late. Chemotherapy becomes the important treatment for liver cancer[6,7]. 5-FU is used extensively in the chemotherapy of hepatocellular carcinoma[8,9]. It is well known that mechanism of the anti-tumor effects is to interfere with DNA or protein synthesis[10]. 5-FU can also induce the apoptosis of different tumor cells, but the mechanism is unclear[11,12]. Oshima T *et al* reported that 5-FU induced iNOS expression and NO produced by gastric cancer cells, and NO participated in antitumor activity in gastric cancer cells. These effects may be mediated by TNF-alpha

production[13]. 5-FU could induce the cytokines such as interleukin-1beta (IL-1beta) and TNF-alpha[14]. Other studies showed that the iNOS expression was stimulated by interleukin-1beta (IL-1beta) and TNF-alpha[15]. So we speculated that the 5-FU might induce the iNOS by induction of cytokines. However, this need to be proved by further studies. We found that 5-FU could induce the expression of iNOS of BEL-7402 cells *in vitro*. When the L-Arg was sufficient, the production of NO increased obviously. It caused the increases of apoptosis and necrosis of BEL-7402 cells. NO increased the chemotherapy sensitivity of BEL-7402 cells to 5-FU[16]. In this study, the expression and activity of iNOS increased after the treatment with 5-FU and L-Arg, and the concentration of NO in the tumor tissue was increased at the same time. This suggests that the endogenous NO plays an important role in the anti-tumor effects of 5-FU and L-Arg.

The present study showed that the high concentration of NO could be cytotoxic for tumor cells or inhibit the tumor growth[17,18]. The high concentration of NO could interfere with the citric acid cycle, and arrest the cell in the S stage[19,20]. More importantly, NO has been shown to bind rapidly with high affinity to ferrous iron (Fe^{2+}). As a consequence, NO can bind easily to free iron, iron within iron-sulphur centres, and iron within hemoproteins[21,22]. NO can cause DNA damage via the generation of peroxynitrite (ONOO-) and N_2O_3[23,24]. One of the consequences of the NO-mediated DNA damage is to trigger p53 accumulation, which can induce apoptosis[25,26]. NO can induce the apoptosis of tumor cell by the regulation of the apoptosis and cell cycle related protein, such as P21, BAX, cyclin D *etc*[27-29]. Nicola *et al* reported that NO mediates chemosensitivity in tumor cells, and Hypoxia-induced drug resistance appears to result, in part, from down-stream suppression of endogenous NO production. These results raise the possibility that administration of small doses of NO mimetics could be used as an adjuvant in chemotherapy[30]. In this study, the expression of P16 and BAX was found correlated with the concentration of NO, suggesting that NO can increase the expression of apoptosis-related gene and antioncogene. The necrotic range and apoptosis index increased more significantly in the 5-FU and L-Arg group than other two groups. We think that the high concentration of NO may kill the tumor cells or induce apoptosis of the tumor cells directly. On the other hand, the production of NO also improved the chemosensitivity of tumor cells to 5-FU.

L-Arg is not an essential amino acid, and it can be synthesized in the liver. However, liver function of liver cancer patients is poor, and the concentration of L-Arg in the body is likely low. When patients are undergoing chemotherapy with 5-FU, added with L-Arg to improve the production of endogenous NO in liver cancer cells may enhance the therapeutic effects and improve the antitumor ability of the patients. L-Arg may be an effective adjuvant for 5-FU.

COMMENTS

Background

Hepatocellar carcinoma is one of the most common malignant tumors in China. 5-FU is used extensively for the treatment of liver cancer. The effects of 5-FU alone is not satisfied. It is necessary to find the effective adjuvant for 5-FU.

Research frontiers

The expression of iNOS in the human liver carcinoma and the effects of endogenous nitric oxide on the BEL-7402 cells have been examined.

Innovations and breakthroughs

In the present study, the effects of endogenous nitric oxide on the liver carcinoma in nude mice have been studied.

Applications

The L-Arg can be used as the adjuvant for 5-FU in the treatment of liver cancer.

Terminology

L-Arginine (L-Arg) is a semi-essential amino acid that possesses numerous useful physiologic properties. NOS can catalyzethe L-Arg to form the nitric oxide. When the L-Arg is sufficient, the production of nitric oxide can be increased.

Peer review

This manuscript is very interesting. The title accurately reflects the major contents of the article. The results provide sufficient experimental evidences from which conclusions are drawn. The conclusions are scientifically reliable and valuable.

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RAPID COMMUNICATION

Two novel germline mutations of MLH1 and investigation of their pathobiology in hereditary non-polyposis colorectal cancer families in China

Chao-Fu Wang, Xiao-Yan Zhou, Tai-Ming Zhang, Ye Xu, San-Jun Cai, Da-Ren Shi

Chao-Fu Wang, Xiao-Yan Zhou, Tai-Ming Zhang, Da-Ren Shi, Department of Pathology, Cancer Hospital of Fudan University, Shanghai 200032; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China
Ye Xu, San-Jun Cai, Department of Abdominal Surgery, Cancer Hospital of Fudan University, Shanghai 200032, China
Supported by the Key Project of Shanghai Medical Subjects, No.05 Ⅲ 004 and Shanghai Pujiang Program, No. 06PJ14019
Correspondence to: Dr. Xiao-Yan Zhou, Laboratory of Molecular Pathology, Cancer Hospital of Fudan University, 270 Dongan Road, Shanghai 200032, China. xyzhou100@yahoo.com
Telephone: +86-21-64175590-3646
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Abstract

AIM: To detect germline mutations of MLH1, and investigate microsatellite instability and expression of MLH1 in tumor tissues of hereditary non-polyposis colorectal cancer (HNPCC) with two novel germline mutations, and further investigate the pathobiology of the two novel mutations of MLH1.

METHODS: RNA was extracted from the peripheral blood of 12 patients from 12 different families that fulfilled the Amsterdam Ⅱ Criteria for HNPCC. Germline mutations of MLH1 were determined by RT-PCR, followed by cDNA sequencing analysis. PCR-GeneScan analysis was used to investigate microsatellite instability with a panel of five microsatellite markers (BAT26, BAT25, D5S346, D2S123 and mfd15), along with immunohistochemical staining to detect the expression of MLH1 protein in two patients' tumor tissues with novel mutations.

RESULTS: Three germline mutations were found in four patients, one of the mutations has previously been reported, but the other two, CGC→TGC at codon 217 of exon 8 and CCG→CTG at codon 581 of exon 16, have not been reported. The two patients' tumor tissues with novel mutations had high-frequency microsatellite instability that showed more than two unstable loci, and both tumors lost their MLH1 protein expression.

CONCLUSION: The two novel germline mutations of MLH1 in HNPCC families i.e. CGC→TGC at codon 217 of exon 8 and CCG→CTG at codon 581 of exon 16, are very likely to have pathological significance.

Key words: Colorectal cancer; Hereditary non-polyposis colorectal cancer; MLH1 gene; Germline mutation; Microsatellite instability; Gene sequencing

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INTRODUCTION

Colorectal cancer remains a serious public health challenge worldwide. According to the different molecular mechanisms, colorectal cancer is divided into two groups: sporadic and genetic. Hereditary non-polyposis colorectal cancer (HNPCC), the most common hereditary colon cancer syndrome[1], is a predominantly inherited disease associated with increased lifetime risk of a range of cancers, including colorectal and endometrial cancers, as well as extracolonic gastrointestinal, genitourinary, ovarian and brain cancers[2-9]. HNPCC development is associated with the functional deficiency of germline MMR genes. Up to now, seven MMR genes have been found, and among these, MLH1 and MSH2 are very closely associated with HNPCC[10-12]. Carriers of germline MMR mutations have a > 80% risk of cancer by the age of 75[13-15]. Twelve patients fulfilling Amsterdam Criteria Ⅱ from China were explored in this study. Two novel MLH1 mutations were detected, and the pathobiology of the novel mutations was investigated.

MATERIALS AND METHODS

Subjects and samples

Twelve patients of Chinese descent from families fulfilling Amsterdam Ⅱ Criteria were selected through the clinic at the Cancer Hospital of Fudan University, Shanghai, China. Personal and family cancer histories were obtained from the patients and participating relatives, and cancer diagnosis was confirmed by reviewing records and pathology reports. Informed consent was obtained from each participant. Three microliters of peripheral blood of each participant was taken. Total RNA of the peripheral blood was extracted using Trizol (Sigma), according to the

Table 1 Sequences and localization of primers used for Amplification of cDNA of MLH1

Sense	Antisense
MLH1-1F (1-18)	MLH1-5R (2198-2175)
CTTGGCTCTTCTGGCGCC	GAGCGCAAGGCTTTATAGACAATG
MLH1-4F (1333-1353)	MLH1-6R (2484-2459)
GCTGAAGTGGCTGCCAAAAAT	TATGTTAAGACACATCTATTTATTTA

manufacturer's instructions. Twenty unrelated volunteers from families without HNPCC were used as controls.

RT-PCR

cDNA was synthesized with RT (Roche Diagnostics), using 0.5 μg total RNA and specific primers complementary to the 3' end of the MLH1 (2484-TATGTTAAGACACAT CTATTTATTTA-2459). cDNA of MLH1 was amplified in two overlapping fragments using primers (Table 1) that generated products of approximately 2000 bp. PCR was performed using Expand Long Template PCR (Roche Diagnostics): 94℃ for 5 min; 10 cycles at 94℃ for 30 s, 59℃ for 30 s, and 68℃ for 3 min; 32 cycles at 94℃ for 30 s, 57℃ for 30 s, 68℃ for 3 min; and a final extension at 68℃ for 7 min[16].

Sequencing

The purified PCR fragments were sequenced directly using a DNA sequencing kit with BigDye Terminators on an ABI3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer' s instructions. The cDNA of MLH1 (2484 bp) was sequenced in six overlapping fragments using primers described in Table 2.

Microdissection and minimal amount of DNA extraction

One 5-μm and four 7-μm paraffin-embedded sections of tumor tissues were deparaffinized. The 5-μm sections were stained with hematoxylin and eosin and served as controls. The 7-μm sections were lightly stained with hematoxylin for microdissection. The microdissection was performed under a dissection microscope with a scalpel. Tumor cells should account for at least 80% of the total cells isolated. The microdissected tissues were transferred directly into an Eppendorf tube with 150 μL cell lysis buffer (0.5 mol/L Tris, 20 mmol/L EDTA, 10 mmol/L NaCl, 10 g/L SDS, 0.5 g/L Proteinase K). The subsequent DNA extraction was performed according to the protocol of the DNA extraction kit (Daxia Biotech, Shanghai, China). Genomic DNA was also extracted from peripheral white blood cells.

Microsatellite instability (MSI) analysis

Matched normal and tumor DNA was investigated with a panel of microsatellite markers (mononucleotide repeats BAT26 and BAT25, dinucleotide repeats D5S346, D2S123 and mfd15)[17]. The primer pairs were synthesized by Shenyou Biotech (Shanghai, China). Each forward primer was labeled with a fluorescent dye at the 5' end to enable the PCR products to be detected by an ABI 310 automated DNA sequencer. After successful amplification, the 2-μL PCR products were mixed with 12.5 μL deionized formamide and 2 μL 350 Rox Sizer. The mixture was denatured, snap-cooled and electrophoresed on an ABI 310 automated DNA sequencer according to the manufacturer's recommendation. The electrophoresis results were analyzed by GeneScan Software (Applied Biosystems). MSI was determined according to the method of Gebert *et al*[18]. Additional peaks (bands) at microsatellite loci in the tumor compared with normal tissue from the same patient were interpreted as MSIs. Cases with MSIs in more than two loci were interpreted as exhibiting high MSI.

Immunostaining for MLH1

A monoclonal antibody against MLH1 (Pharmingen, San Diego, CA, USA) was prepared at a 1:40 dilution. The antibody was detected by the EnVision method. Diminished expression of MLH1 in cancer tissues was demonstrated when there was complete absence of detectable nuclear staining of neoplastic cells. Infiltrating lymphocytes, as well as normal colonic crypt epithelium next to the tumor area, served as internal positive controls[19,20].

RESULTS

Germline mutations of MLH1

Four germline mutations were detected at three different sites of MLH1, involving four patients, which were at 649 codon 217 exon 8: CGC→TGC in family H2, at 1742 codon 581 exon 16: CCG→CTG in family H31, and at 1151 codon 384 exon 12: GTT→GAT in family H109 and H114. All three were missense mutations (Table 3, Figures 1 and 2). Their polymorphism possibilities were excluded by visiting the mutation database of MMR genes (www.INSIGHT-group.org). The mutation in families H109 and H114 has been reported to be pathogenic, while the mutations in H2 and H31 are not. The three abnormalities in MLH1 were not found in the control group.

MSI analysis

Four loci in BAT25, BAT26, D2S123 and D5S346 showed MSI in the tumor tissue of the patient from the H2 family, and four loci in BAT25, BAT26, D2S123 and Mfd15 showed MSI in the tumor tissue of the patient from the H31 family. According to the criteria above for MSI, the tumor tissues of the two patients had high MSI (Figure 3).

Immunohistochemistry of MLH1

There was no expression of MLH1 protein in the tissues of the two patients. As a control, MLH1 protein was detected in infiltrating lymphocytes and the normal colonic crypt epithelium next to the tumor area in the patient from the H2 family, and in the stromal cells in the patient from the H31 family (Figures 4 and 5).

DISCUSSION

Colorectal cancer is one of the most common malignant tumors; furthermore, its incidence is increasing continuously. HNPCC, genetic colorectal cancer, accounts for about 10% of all colorectal cancer. Compared with sporadic colorectal cancer, HNPCC shows special characteristics associated with its molecular mechanisms

Table 2 MLH1 primers used for sequencing of cDNA

	Sense		Antisense
MLH1-1F	CCTGGCTCTTCTGGCGCC	MLH1-1R	CTTTTCTCCTCGTGGCTATGTTGT
MLH1-2F	ATGTGCTGGCAATCAAGGGA	MLH1-2R	GGTGCACATTAACATCCACATTCT
MLH1-3F	CCAAAAACACACACCCATTCCT	MLH1-3R	CCTTTGTTGTATCCCCCTCCA
MLH1-4F	GCTGAAGTGGCTGCCAAAAAT	MLH1-4R	CATCTTCCTCTGTCCAGCCACTC
MLH1-5F	TTGCCATGCTTGCCTTAGATAGTC	MLH5R	GAGCGCAAGGCTTTATAGACAATG
MLH1-6F	GCTCCATTCCAAACTCCT	MLH1-6R	TATGTTAAGACACATCTATTTATTTA

Table 3 Germline mutations of MLH1

Families	Genes	Exon	Codons affected	DNA change	Amino acid change	Mutation types
H2	MLH1	8	217	C→T, at 649	Arg→Cys	Missense
H31	MLH1	16	581	C→T, at 1742	Pro→Leu	Missense
H109	MLH1	12	384	T→A, at 1151	Val→Asp	Missense
H114	MLH1	12	384	T→A, at 1151	Val→Asp	Missense

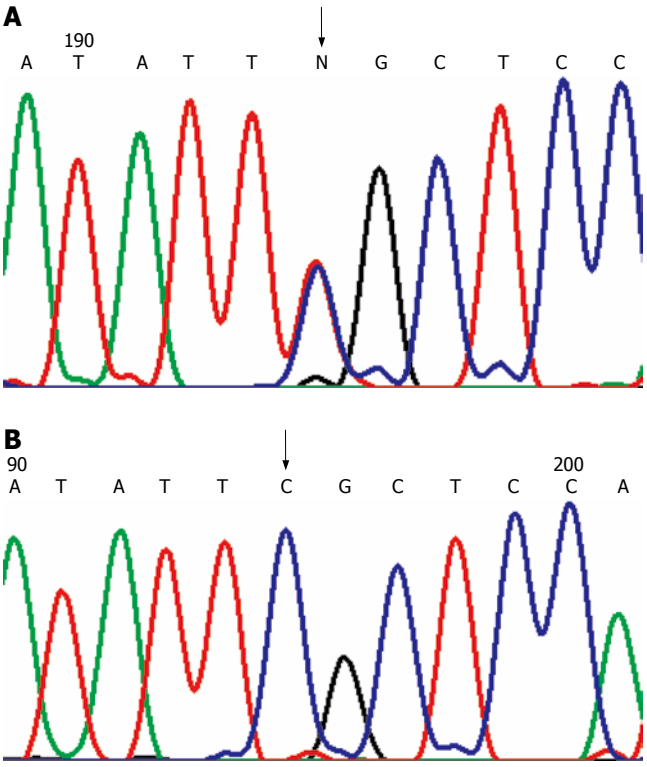

Figure 1 Missense mutations. (**A**): MLH1 mutation in H2 family at 649 codon 217 exon 8: CGC→TGC. The arrow shows the site of the mutation; (**B**): Wild-type sequence. The arrow shows the corresponding site.

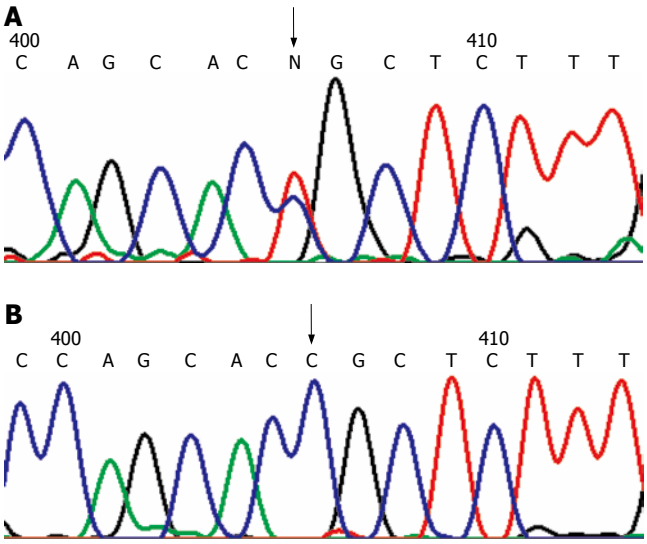

Figure 2 Missense mutation. **A**: MLH1 mutation in H31 family at 1742 odon 581 exon 16: CCG→CTG. The arrow shows the site of the mutation; **B**: Wild-type sequence. The arrow shows the corresponding site.

and clinic features. HNPCC, as the most common hereditary colon cancer syndrome, is characterized by early onset of colorectal cancer, location of tumors in the proximal colon, and an increased risk of neoplasms of extracolonic organs, including endometrium, stomach, urothelium, small intestine, and ovary, multiple metachronous colorectal cancer[21-24], and better prognosis than that in sporadic cases[25-30]. Development of HNPCC is closely associated with deficiency or loss of MMR gene function. Identification of MMR gene germline mutations can have direct clinical implications in counseling and management of HNPCC families[31].

HNPCC has gained worldwide recognition, and several developed countries, such as the United States, Germany, Finland and the Netherlands, have established HNPCC genetic institutions, and several clinical criteria for the diagnosis of concerned families have been suggested. However, at present, there are only are a few institutions in China that are engaged in research on HNPCC. Our hospital collaborative group on HNPCC has been involved in the field for a few years, and has set up an HNPCC database. We detected the 12 random samples from our database that fulfilled Amsterdam Criteria Ⅱ, using an mRNA-based sequencing technique, and three germline mutations of MLH1 were found. All three were missense mutations, two of which have not been reported previously. Diagnosis of HNPCC was based on finding the pathological germline mutation in MMR gene. In the present series, difficulties in the assessment of pathogenicity were mostly associated with missense mutations. Peltomaki *et al* thought the missense mutation meeting the following criteria was pathogenic (1)

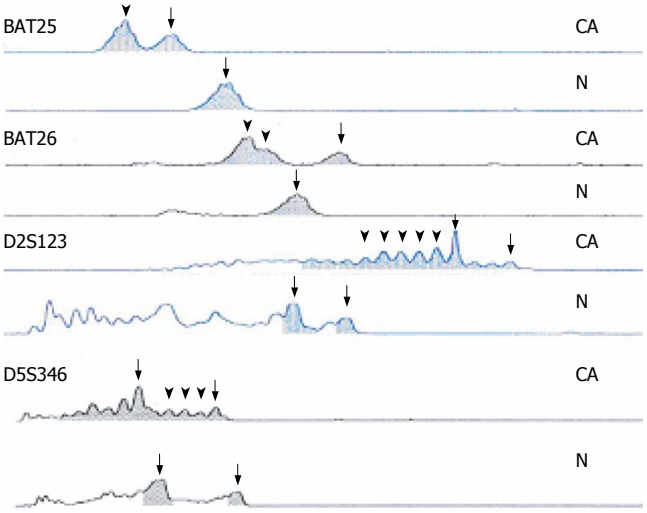

Figure 3 Four sites of MSI in H2 family. CA represents the tumor tissue, N represents the control tissue, the arrow-heads show new waves, and the arrows show wild ones.

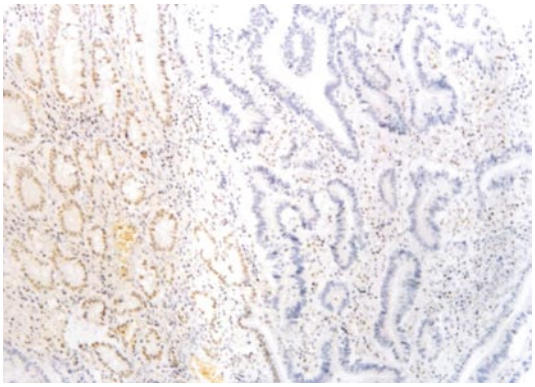

Figure 4 MLH1 protein in H2 was negative in the tumor glands (right), and positive in the mucous glands next to the tumor tissue (EnVison, × 200).

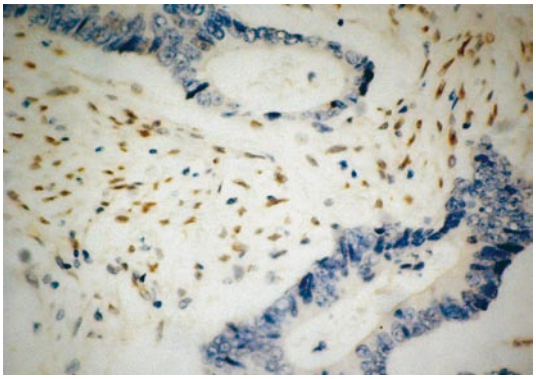

Figure 5 MLH1 protein in H31 was negative in the tumor glands, and positive in the stroma cells (EnVison, × 400).

that it led to a nonconservative aminoacid change, (2) that the involved codon was evolutionarily conserved, (3) that the alteration did not occur in the normal population, and (4) that it cosegregated with the disease phenotype[11]. The patient in the H2 family was affected by colorectal cancer at 36 years of age, his mother was affected by endometrial cancer at 54 years of age, and one of his aunts was affected by colorectal cancer at 48 years of age. The patient in the H31 family was affected by colorectal cancer at 39 years of age, her father was affected by colorectal cancer at 50 years of age, and her uncle was affected by gastric carcinoma at 57 years of age. Their family histories suggested co-segregation of the mutations with the disease. Both of the novel mutations lead to amino acid changes, and the changed amino acids belong to the non-conserved ones. According to the criteria above, we estimated that the two novel mutations were pathogenic. In order to further evaluate the mutations, DNA were isolated from the two patients' tumor tissues, and GeneScan was employed for MSI analysis, and immunohistochemistry was used to detect the expression of MLH1 protein in tumor tissues[32,33]. The tumor tissues of the two patients showed high MSI and lack of MLH1 protein expression. Based on the above results, we concluded that the two novel mutations were very likely pathogenic.

Differentiating HNPCC from sporadic colorectal cancer has practical clinical value, and the identification of HNPCC depends on the detection of germline mutations of the MMR gene. More MMR genes should be investigated besides MLH1, and when a novel mutation is found, its pathogenic evaluation should be carried out so that more HNPCC can be identified.

COMMENTS

Background

Hereditary non-polyposis colorectal cancer (HNPCC) is one of the most common autosomal dominantly inherited cancer syndromes and accounts for 10% of all colorectal cancer. HNPCC shows its own characteristics associated with its molecular mechanisms, clinical features, method of treatment, and management of HNPCC families. It has gained worldwide recognition, and the International Collaborative Group on Hereditary Non-polyposis Colorectal Cancer (ICG-HNPCC) was founded in 1990. At present, there are a few ways to screen HNPCC families; however, only the pathological germline mutation in the mismatch repair (MMR) gene can be used for identifying HNPCC families.

Research frontiers

Development of HNPCC is closely associated with deficiency or loss of function of MMR genes. At least 5 MMR genes, MLH1, MSH2, MSH6, PMS1, and PMS2, have been implicated in HNPCC. Genetic linkage analysis showed that germline mutations of MLH1 and MSH2 account for nearly 90% of all the germline mutations found in HNPCC. Germline mutations of MLH1 were detected in this study.

Innovations and breakthroughs

There are a few ways to screen HNPCC families, however, the most specific method is to detect germline mutations of MMR genes. In present study, a new method, RNA-based sequencing analysis, was used. Two novel germline mutations in MLH1 were found, and their possible pathobiology was investigated by PCR-GeneScan analysis and immunohistochemical staining.

Applications

Identification of pathological germline mutations in MMR genes is the gold standard for HNPCC. Differentiating HNPCC from sporadic colorectal cancer has direct clinical implications for counseling and management of HNPCC family members.

Peer review

HNPCC is different from sporadic colorectal cancer(SCRC), and to differentiate HNPCC from SCRC owns practical clinical implications. The study detected germline mutations of MLH1 with a new method, and investigated the pathobiology of the detected novel mutations in MLH1. The study is a valuable contribution with potential clinical importance.

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RAPID COMMUNICATION

Effects of short-term application of low-dose growth hormone on trace element metabolism and blood glucose in surgical patients

Kun Qian, Zhi Wan, Lang-Song Hao, Ming-Ming Zhang, Yong Zhou, Xiao-Ting Wu

Kun Qian, Lang-Song Hao, Ming-Ming Zhang, Yong Zhou, Xiao-Ting Wu, Department of General Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Zhi Wan, Pediatrics, West China Second Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Correspondence to: Professor Xiao-Ting Wu, Department of General Surgery, West China Hospital, Sichuan University, 37 Guo Xue Road, Chengdu 610041, Sichuan Province, China. hxjsqk@163.com
Telephone: +86-13980523523 Fax: +86-288-5422483
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Abstract

AIM: To investigate the effects of short-term application of low-dose growth hormone on trace element metabolism and blood glucose in surgical patients

METHODS: A total of 48 consecutive patients undergoing abdominal operations were randomized to receive either subcutaneous rhGH (0.15 IU/kg) or placebo (menstruum) injections daily for 7 d after surgery. The two groups had similar nutrition intake. Blood, feces, urine and drain samples were collected to measure zincum, cuprum and ferrum as well as glucose levels. Accumulative intake, excretion and balance of zincum, cuprum and ferrum, apparent absorption (AA) and apparent utilization (AU) of zincum, cuprum and ferrum, blood glucose levels and adverse events were estimated.

RESULTS: There were no differences in accumulative intake and drain excretion between the two groups. The feces excretion and accumulative excretion of cuprum were lower in the rhGH group ($P < 0.05$). The urinary excretion of zincum, cuprum and ferrum was all significantly decreased in the rhGH group ($P < 0.05$) and the accumulative balance of zincum, cuprum and ferrum was improved compared with the placebo group ($P < 0.05$). AA of cuprum in the rhGH group was almost twice as much as the placebo group ($P < 0.05$), and AU of zincum, cuprum and ferrum was all improved in the rhGH group ($P < 0.05$). The mean blood glucose level was significantly higher in the rhGH group than in the placebo group from d 3 to d 6 after operation ($P < 0.05$).

CONCLUSION: Postoperative low-dose rhGH treatment improves the retention of zincum, cuprum and ferrum and decreases the excretion of zincum, cuprum and ferrum, improves the balance of zincum, cuprum and ferrum, and promotes the AA and AU of zincum, cuprum and ferrum. rhGH can be well tolerated without significant adverse effects and the blood glucose level can be well controlled.

Key words: Growth hormone; Metabolism; Trace elements; Zincum; Cuprum; Ferrum

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INTRODUCTION

Patients undergoing abdominal surgery often suffer from severe trauma or infection caused by catabolic responses[1], which cannot be prevented by conventional parenteral or enteral nutrition formulas[2,3]. Administration of recombinant human growth hormone (rhGH) has been shown to significantly maintain the nitrogen balance and increase the protein synthesis in surgical patients receiving either parenteral or enteral nutrition[4-7]. Most of such studies paid more attention to nitrogen balance and protein metabolism changing associated with rhGH treatment. However, there are few studies focusing on the effects of rhGH on trace element metabolism in patients. The present study was to evaluate the effects of rhGH on trace element metabolism and blood glucose levels in selective abdominal surgical patients.

MATERIALS AND METHODS

Patients

The study was conducted in accordance with the guidelines for Good Clinical Practice and the provisions of the Declaration of Helsinki in 1995 as revised in Edinburgh 2000, and approved by the Ethical Review Committee of West China Hospital. Only those who consented to

participate in the study after explanation of the objectives and protocol were included in the study. Signed, informed consent was obtained from all patients and their close relatives.

Forty-eight adult patients were enrolled in the study and all met the following criteria: undergoing a selective abdominal operation, aged 18-75 years, willing and being able to comprehend the protocol and give written informed consent. Exclusion criteria were as follows: severe bacterial infection, liver and renal dysfunction, previous or current treatment with corticosteroids, diabetis mellitus or fasting glucose levels greater than or equal to 7.0 mmol/L, metabolic diseases, gestation, severe malnutrition (serum albumin < 21 g/L), tumor recrudescence or metastasis.

Study design

The study was a randomized prospective double-blind, placebo-controlled clinical trial. Eligible patients were randomly assigned to rhGH group or placebo group (24 each group). The randomization codes were prepared with the random number table according to the design of a computer. Patients, surgeons and nursing staff members remained blind to the allocation status of the study drugs throughout the experiment.

After operation, all patients received continuous combined intravenous or/and enteral nutrition. The daily total caloric requirement was 20 kcal/kg and total nitrogen requirement was 140 mg of nitrogen/kg. Parenteral nutrition (PN) solution was prepared aseptically using commercially available products, including vitamins, trace elements and electrolytes (Addamel, Vitlipid, Soluvit and Glycophos; Fresenius Kabi Deutschland Gmbh, Bad Homburg, Germany). Amino acid injections were provided as 8.5% and 11.4% Novamin (Fresenius Kabi Deutschland Gmbh). Energy calories were provided with glucose and fat emulsion injections (50% glucose and 20% Lipovenos® MCT; Fresenius Kabi Deutschland Gmbh). All the nutrients were given in all-in-one bag. Enteral nutrition (EN) emulsion (Fresenius Kabi Deutschland Gmbh) was provided orally or via a nasogastric tube with a continuous perfusion pump.

Postoperatively, patients received general intravenous infusion with only glucose on d 1; PN provided only half of total caloric and nitrogen requirement on d 2 and all of total requirement on d 3; on d 4, PN provided 2/3 of total requirement and EN provided another 1/3; on d 5, PN provided 1/3 of total requirement and EN provided 2/3; only EN emulsion was given from d 6 to d 9.

From d 3 to d 10 post operation, patients were randomly assigned to receive identical-looking treatments consisting of either rhGH (JINTROPIN®, 0.15 mg/kg) or menstruum injection (1 mL, consisting of glycin, mannitol, lactose and sodium bicarbonate) subcutaneously once daily. rhGH and placebo were provided by GeneScience Pharmaceutical Co. Ltd, Changchun, China.

Laboratory tests

Blood samples were drawn from each patient before operation to measure baseline values and on d 3 and 10 after operation to study the rhGH effect. Complete blood cell count was estimated by the XE-2100 (Sysmex, Kobe, Japan). Plasma glucose, serum urea nitrogen, creatinine, bilirubin, alanine aminotransferase, alkaline phosphatase, total protein, albumin and electrolytes were estimated using an Olympus AU5400 autoanalyser (Olympus, Tokyo, Japan).

Table 1 Baseline characteristics of patients (mean ± SD)

Variable	Placebo (n = 24)	rhGH (n = 24)	P
Age (yr)	58.50 ± 9.35	59.08 ± 10.93	0.789
	39-75	35-74	
Sex (femal/male)	11/13	9/15	0.558
Weight (kg)	57.90 ± 8.42	56.19 ± 11.83	0.567
Height (cm)	162.42 ± 6.92	162.88 ± 7.16	0.823
Sepsis score	0.79 ± 0.98	0.67 ± 0.87	0.742
Operation position, n (%)			
Resection of stomach	6 (25)	5 (20.8)	0.297
Resection of colon	5 (20.8)	7 (29.2)	
Resection of rectum	12 (50)	9 (37.5)	
Others	1 (4.2)	3 (12.5)	
Acumulative intakes of energy (10^3 kcal)	7.98 ± 0.67	7.76 ± 0.76	0.292
Acumulative intakes of nitrogen (g)	54.84 ± 5.23	53.91 ± 7.05	0.605

Trace element balance

Daily trace element input was assumed to be the trace element contents (zincum, cuprum, ferrum) in PN/or EN solution given. Daily trace element loss was assessed by collecting 24-h output and measuring the trace element contents in feces, urine and drains. Accumulated trace element balance was calculated by subtracting 7 d trace element output from 7 d trace element input. Trace element contents in samples were determined by the inductively coupled plasma atomic emission spectrometry (ICPAES) and estimated by the IRIS ADVANTAGE 1000 (Thermo Elemental, USA).

Statistical analysis

All data were assessed for normality of distribution and equality of variance. Student's t-test and multiple correlation analysis were used to compare normal distribution of data. Data are presented throughout as mean ± SD. All data analyses were performed using the program SPSS 11.5 for Windows. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

There was no difference in baseline characteristics between the two groups (Table 1).

Accumulative intake, excretion and balance of zincum, cuprum and ferrum

As shown in Table 2, there were no differences in accumulative intake and drain excretion between the two groups. The feces excretion and accumulative excretion of cuprum were lower in the rhGH group. The urinary excretion of zincum, cuprum and ferrum was

Table 2 Accumulative intake, excretion and balance of zincum, cuprum and ferrum

Test	Zincum		Cuprum		Ferrum	
	Placebo (n = 24)	rhGH (n = 24)	Placebo (n = 24)	rhGH (n = 24)	Placebo (n = 24)	rhGH (n = 24)
Accumulative intake (mg)	65.79 ± 6.38	71.92 ± 6.96	10.01 ± 0.85	10.82 ± 0.92	65.08 ± 8.50	73.25 ± 9.29
Urinary excretion (mg)	36.74 ± 5.76	32.51 ± 5.55[a]	0.37 ± 0.08	0.28 ± 0.05[a]	18.65 ± 5.99	12.10 ± 3.92[a]
Feces excretion (mg)	19.14 ± 6.49	18.07 ± 7.50	4.21 ± 1.00	1.74 ± 0.95[a]	22.38 ± 9.82	19.65 ± 3.71
Drain excretion (mg)	2.74 ± 0.48	2.48 ± 0.62	0.84 ± 1.00	0.61 ± 0.05	14.83 ± 4.05	16.69 ± 3.32
Accumulative excretion (mg)	58.62 ± 8.69	53.06 ± 9.35	5.42 ± 1.42	2.63 ± 0.95[a]	55.86 ± 12.19	48.44 ± 6.34
Accumulative balance (mg)	7.17 ± 5.90	18.86 ± 6.24[a]	4.59 ± 1.33	8.19 ± 0.28[a]	9.22 ± 8.74	24.81 ± 6.79[a]

[a]P < 0.05 *vs* placebo group.

Table 3 Comparison between apparent absorption (AA) and apparent utilization (AU) of zincum, cuprum and ferrum (mean ± SD)

Test	Zincum		Cuprum		Ferrum	
	Placebo (n = 24)	rhGH (n = 24)	Placebo (n =24)	rhGH (n = 24)	Placebo (n = 24)	rhGH (n = 24)
Accumulative intake (mg)	65.79 ± 6.38	71.92 ± 6.96	10.01 ± 0.85	10.82 ± 0.92	65.08 ± 8.50	73.25 ± 9.29
Accumulative intake of EN (mg)	46.29 ± 6.38	52.42 ± 6.96	6.17 ± 0.85	6.98 ± 0.92	61.72 ± 8.50	69.89 ± 9.29
Urinary excretion (mg)	36.74 ± 5.76	32.51 ± 5.55	0.37 ± 0.08	0.28 ± 0.05	18.65 ± 5.99	12.10 ± 3.92
Feces excretion (mg)	19.14 ± 6.49	18.07 ± 7.50	4.21 ± 1.00	1.74 ± 0.95[a]	22.38 ± 9.82	19.65 ± 3.71
Apparent absorption (%)	59.09 ± 10.56	66.25 ± 11.07	31.80 ± 13.78	75.21 ± 13.69[a]	64.94 ± 11.72	71.87 ± 4.00
Apparent utilization (%)	15.21 ± 5.43	30.02 ± 6.23[a]	54.35 ± 8.87	81.36 ± 8.57[a]	37.31 ± 6.85	56.34 ± 6.99[a]

[a]P < 0.05 *vs* placebo group.

Table 4 Comparison of blood glucose levels

Test	D1	D3	D4	D5	D6	D7	D8	D9
Glucose (mmol/L)								
Placebo	5.26 ± 1.09	5.68 ± 1.33	5.81 ± 1.56	5.84 ± 1.48	5.95 ± 2.34	6.01 ± 2.64	5.66 ± 2.03	5.70 ± 1.89
rhGH	5.14 ± 0.64	6.71 ± 1.93[a]	7.17 ± 1.86[a]	8.28 ± 2.30[a]	7.68 ± 2.15[a]	7.29 ± 2.93	6.40 ± 2.00	6.20 ± 2.13

[a]P < 0.05 *vs* placebo group.

Table 5 Main adverse events

Event	Placebo	rhGH
Hyperglycemia	4	23[a]
Tetter	1	0
Sepsis	0	0
Infection	2	3
Death	0	0

[a]P < 0.05 *vs* placebo group.

all significantly decreased in the rhGH group and the accumulative balance of zincum, cuprum and ferrum was significantly improved compared with the placebo group.

Apparent absorption (AA) and apparent utilization (AU) of zincum, cuprum and ferrum

The cuprum was mostly excreted *via* feces. AA of cuprum in the rhGH group was almost twice as much as that in the placebo group, and AU of zincum, cuprum and ferrum was improved in the rhGH group (Table 3).

Blood glucose levels and adverse events

The main adverse effects seen during the study are summarized in Tables 4 and 5. The mean blood glucose level was significantly higher in the rhGH group than in the control group from d 3 to d 6 after operation (Table 4). Twenty-there patients in the rhGH group experienced hyperglycemia and 5 of them required insulin treatment (Table 5). Furthermore, 3 patients had other mild adverse events (1 with edema, 1 with tetter and 1 with fever). In the placebo group, 3 of 4 patients presenting hyperglycemia required insulin treatment. Five placebo-treated patients experienced mild electrolyte imbalance, which was not related the trial drug used. There was no significant difference in complete blood cell count, liver and renal function, body weight and daily clinical parameters such as temperature, blood pressure, and pulse, between the two groups.

DISCUSSION

Many attempts have been made to reverse the catabolic changes that occur in postoperative patients. Conventional nutrition support is unable to provide adequate nutritional supplements to increase or even maintain body proteins and trace elements in hypercatabolic response conditions[8-10]. Recent studies indicate that rhGH can stimulate body protein synthesis and produce nitrogen-spacing effects[11-13]. However, the impact of rhGH on body trace elements and blood glucose has not been

investigated in patients receiving PN or EN following selective gastrointestinal surgery[14-16]. In the present experiments, we studied the effects of rhGH on trace element metabolism and blood glucose. The number of patients enrolled in the study was based on previous experiments and the dosage of rhGH used[7,17,18].

Massive trace elements are lost after selective operation because of decreased intake, loss from wound surface, redistribution in the body and increased urinary excretion[19,20]. Even supplying adequate nutritional support cannot prevent such a massive loss of trace elements. Zincum, cuprum and ferrum are very important trace elements in the human body and can sensitively reflect changes in gastric diseases[21]. In this study, low-dose rhGH treatment reduced the urinary excretion of zincum, cuprum and ferrum, thus improving their accumulative balance compared with the placebo group. Meanwhile, the apparent absorption and utilization of zincum, cuprum and ferrum in the rhGH group were also increased. However, the AU of zincum in the rhGH group (30.02%) was almost two times higher than that in the placebo group. The AU of cuprum and ferrum in the rhGH group was also about 1.5 times higher than that in the placebo group. These data indicate that low-dose rhGH treatment can reduce the excretion of zincum, cuprum and ferrum, increase their utilization, and maintain the retention and balance of zincum, cuprum and ferrum.

Changes in zincum, cuprum and ferrum metabolism are mainly associated with protein synthesis and breakdown. Since proteins are carriers of many trace elements, rhGH may also improve protein synthesis, reduce protein breakdown, promote recovery of intestinal mucosa, increase mucosa thickness, improve intestinal barrier function, and increase absorption of trace elements[22-24]. In our study, the apparent absorption and utilization of zincum, cuprum and ferrum were improved in the rhGH group.

It was reported that GH given during sepsis can impair immune function and result in hyperglycemia, which may explain why acute critically ill patients do not benefit from GH treatment[25,26]. However, selective surgical patients can safely administer GH after the acute inflammatory response stage. rhGH treatment was generally well tolerated with no serious adverse events occurred in our trial. No death occurred in the GH-treated group, confirming its safety. These results are contrary to the increased mortality among critically ill patients treated with GH[25]. We hypothesize that this discrepancy might be due to the difference in study patients. In our study, the patients were selective surgery subjects. rhGH given during the response to stress leads to uncontrolled systemic inflammation in Takala's study[25].

The main adverse event of rhGH treatment is hyperglycemia. Insulin resistance caused by rhGH plays an important role in the elevation of blood glucose. Other reasons include nutrition support and systemic inflammation syndrome[27,28]. In our study, hyperglycemia caused by rhGH administration was mild and controlled by insulin. Considering the difference between critically ill patients and selective surgery patients, rhGH seems to be well tolerated after operation.

Since our study included 14 cancer patients in the rhGH group, the potential tumor-promoting effect of GH should be addressed. In animal models, the role of rhGH administration in promoting tumor recurrence is controversial[29-31]. It was reported that GH could promote host growth selectively and inhibit tumor metastasis[32,33]. Only two trials have assessed the impact of GH on tumor recurrence in humans. Based on 2632 adverse events, the National Cooperative Growth Study analyzed the recurrence of brain tumors in patients receiving long-term GH replacement, showing that there is no evidence that GH increases tumor recurrence[34]. Only one study has investigated the impact of short-term treatment with three different doses of GH on long-term tumor recurrence in postoperative cancer patients[35], finding that 35% rhGH-treated patients have tumor recurrence in comparison to 44% placebo-treated patients. Based on the above two studies, we believe that when complete resection and appropriate antineoplastic treatment are administered, cancer patients can safely receive short-term GH treatment.

In conclusion, postoperative low-dose rhGH treatment improves the retention and decreases the excretion of zincum, cuprum and ferrum, increases the balance and promotes their apparent absorption and utilization. rhGH is also well tolerated with no significant adverse effects and can control the blood glucose level. A larger trial is required to determine the clinical endpoints such as infection, morbidity, mortality and tumor recurrence.

COMMENTS

Background

Patients undergoing abdominal surgery often suffer from severe trauma or infection caused by catabolic responses, which cannot be prevented by conventional parenteral or enteral nutrition formulas. Administration of recombinant human growth hormone (rhGH) has been shown to significantly maintain the nitrogen balance and increase the protein synthesis in surgery patients receiving either parenteral or enteral nutrition.

Research frontiers

Many studies paid attention to nitrogen balance and protein metabolism associated with rhGH treatment. However, there are few studies focusing on the effects of GH on trace element metabolism in patients. This study was to evaluate the effects of rhGH on trace element metabolism and blood glucose levels in selective abdominal surgical patients.

Innovations and breakthroughs

This study evaluated the effects of rhGH on trace element metabolism and blood glucose levels in selective abdominal surgical patients. Postoperative low-dose rhGH treatment improves the retention of zincum, cuprum and ferrum, and decreases their excretion, increases their balance and promotes their apparent absorption and utilization. rhGH is well tolerated with no significant adverse effects and can control the blood glucose level.

Applications

The results of this study will promote the short-term low-dose rhGH application in clinical practice. Hyperglycemia is the main adverse event of short-term low–dose rhGH treatment.

Terminology

Biosynthetic human growth hormone, also referred to as recombinant human growth hormone, is also called somatropin and abbreviated as rhGH.

Peer review

This is the first study analyzing the effects of growth hormone on trace element metabolism and significantly adds our knowledge on the beneficial effect of short-term GH application.

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RAPID COMMUNICATION

Relationship between survivin expression and recurrence, and prognosis in hepatocellular carcinoma

Chao-Ping Ye, Cheng-Zhi Qiu, Zhong-Xin Huang, Qi-Chen Su, Wei Zhuang, Rui-Lan Wu, Xin-Feng Li

Chao-Ping Ye, Cheng-Zhi Qiu, Wei Zhuang, Xin-Feng Li, Department of Hepatobiliary Surgery, Second Affiliated Hospital of Fujian Medical University, Quanzhou 362000, Fujian Province, China
Zhong-Xin Huang, Qi-Chen Su, Rui-Lan Wu, Department of Pathology, Second Affiliated Hospital of Fujian Medical University, Quanzhou 362000, Fujian Province, China
Correspondence to: Chao-Ping Ye, Department of Hepatobiliary Surgery, Second Affiliated Hospital of Fujian Medical University, Quanzhou 362000, Fujian Province, China. xulina9995@yahoo.com.cn
Telephone: +86-595-22780153 Fax: +86-595-22793591
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Abstract

AIM: To study the expression of the inhibitor of apoptosis protein survivin in hepatocellular carcinoma (HCC), and its correlation with clinicopathological factors, cell proliferation, recurrence and prognosis after hepatectomy.

METHODS: Immunohistochemical staining of survivin and Ki-67 was performed by the standard streptavidin-peroxidase technique on paraffin sections of 55 cases of HCC.

RESULTS: The positive rate of survivin in HCC was 52.7% (29/55). Significant correlation was found between survivin expression with portal vein thrombi and intrahepatic matastasistic nodes ($P < 0.05$). The recurrent rate in survivin-positive HCC was significantly higher than that in survivin-negative HCC after hepatectomy, the 1- and 3-year survival rate in patients with survivin-positive tumors was significantly lower than that in patients with survivin-negative tumors (58.62 and 10.34% *vs* 76.92 and 30.77%, $P < 0.05$, log-rank test). The proliferation index (Ki-67) in survivin-positive HCC (33.83% ± 18.90%) was significantly higher than that in survivin-negative HCC (19.60% ± 19.35%) ($P < 0.05$).

CONCLUSION: Survivin may play an important role in progression of HCC by promoting cell proliferation, and may be positively correlated with high risk of disease recurrence and poor prognosis in HCC. Its expression may serve as a prognostic factor for patients with HCC after hepatectomy.

Key words: Hepatocelluar carcinoma; Survivin; Proliferation index; Prognosis; Immunohistochemistry

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INTRODUCTION

Although surgical resection is the most important method for hepatocellular carcinoma (HCC), the recurrent rates may be as high as 50% at 2 years after hepatectomy[1]. The recurrence of HCC may be related to a variety of factors, including biological markers. Molecular prognostic markers are likely to be of greatest benefit in the effective management of patients with HCC, however, these factors have not yet been sufficiently defined in patients with a high risk of cancer recurrence.

Survivin is a recently described member of the family of inhibitor of apoptosis proteins (IAPs). Recently, it has been shown that survivin is strongly associated with apoptosis, cell proliferation and cell-cycle control[2-5]. Survivin plays a crucial role in the genesis and progression of malignancy and is an important prognostic parameter in tumors[6-10]. This study investigated the expression of survivin in HCC and its correlation with clinicopathological factors, cell proliferation and prognosis.

MATERIALS AND METHODS

Materials

Tissue samples were obtained between December 2000 and December 2003 from 55 patients with HCC (41 men, 14 women; 24-74 years old, mean age, 48.65 years). There were 27 patients with stage Ⅰ-Ⅱ, and 28 with stage Ⅲ-Ⅳ cancer. None of the patients received radiotherapy, chemotherapy or immunotherapy before surgery.

Reagents

Rabbit anti-human survivin polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-human Ki-67 monoclonal antibody

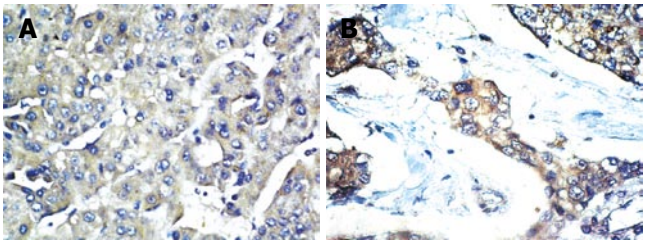

Figure 1 **A:** Positive expression of survivin in HCC (SP, × 200); **B**: Positive expression of survivin in HCC (SP, × 400).

(MBI.1), streptavidin-peroxidase (SP) staining kit and diaminobenzidine (DAB) kit were supplied by Maixin-Bio, Fuzhou, China.

Methods

Expression of survivin and Ki-67 was detected using SP immunohistochemistry. Briefly, after deparaffinization and rehydration, antigen retrieval was accomplished by incubation in 0.01 mol/L citric acid buffer (pH 6.0), boiling for 1 min in a pressure cooker, and cooling and washing in tap water. The sections were incubated with hydrogen peroxide for 10 min and washed in PBS. Non-specific reactions were blocked by incubation in a solution containing normal serum. The sections were incubated with a primary antibody (anti-survivin or Ki-67 antibody) overnight at 4℃. The working dilution of anti-survivn antibody was 1:100. The sections were rinsed with PBS, and then incubated for 10 min at room temperature with biotinylated secondary antibody. After washing, streptavidin-biotin complex conjugated to horseradish peroxidase was applied for 10 min at room temperature. After three rinses with PBS, the sections were incubated with DAB, rinsed with distilled water, counterstained with hematoxylin, and dehydrated and coverslipped. The sections were prepared for microscopy. Colorectal cancer tissues were used as a positive control. As a negative control, PBS was used to replace primary antibody.

Scoring criteria for survivin expression

Intensity and percentage of positive cells were used to evaluate each tissue section. The mean percentage of positive tumor cells and normal epithelial cells in at least five areas at × 400 magnification was determined and assigned to one of five categories: 0, < 5%; 1, 5%-24%; 2, 25%-49%; 3, 50%-74%; and 4, ≥ 75%. The intensity of survivin immunostaining was scored as 0 (achromatic), 1 (light yellow), 2 (yellow), and 3 (brown). The percentage of positive cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores < 1 were defined as negative; all others were defined as positive.

Determination of the Ki-67 proliferation index

At least five high-power fields were chosen randomly in each section, and 500 cells were counted for each field. The Ki-67 proliferation index was defined as the number of Ki-67-positive nuclei divided by the total number of colorectal cancer cells counted, and was expressed as a percentage.

Table 1 Correlation between survivin expression and clinicopathology in HCC *n* (%)

Clinicopathological factor		*n*	Survivin expression		*P* value
			positive	negative	
Sex	Male	41	23	6	0.392
	Female	14	6	8	
Age (yr)	≤ 55	40	22	18	0.581
	> 55	15	7	8	
Tumor site	Right lobe	30	14	16	
	Left lobe	20	12	8	0.615
	Whole liver	5	3	2	
HBsAg	Positive	42	21	21	0.467
	Negative	13	8	5	
Differentiation	Moderate to well	40	23	17	0.247
	Poor	15	6	9	
Intrahepatic metastatic nodes	(+)	21	16	5	0.006
	(–)	34	13	21	
Portal vein thrombi	(+)	14	12	2	0.004
	(–)	41	17	24	
Tumor capsule	(+)	22	12	10	0.825
	(–)	33	17	16	
Tumor size (cm)	≤ 5	16	8	8	0.795
	> 5	39	21	18	
AF P(μg/L)	< 400	19	7	12	0.086
	≥ 400	36	22	14	
Hepatocirrhosis	(+)	37	21	16	0.391
	(–)	18	8	10	
Tumor stage	Ⅰ-Ⅱ	27	14	13	0.898
	Ⅲ-Ⅳ	28	15	13	

Statistical analysis

The survival curves were assessed by the Kaplan-Meier method and compared by a log-rank test. The χ^2 test was performed for enumeration data comparison, and the *t* test was used for comparison of measurement data. $P < 0.05$ was considered statistically significant. All data analysis was performed with commercially available statistical analysis software packages (SSPS 11.5, SSPS, Chicago, IL, USA).

RESULTS

Relationship between expression of survivin and clinical pathology

Survivin protein expressed as brown-yellow particles in the cytoplasm after staining, and only one expressed both in the cytoplasm and nucleus after staining. The positive staining rate for survivin in the cytoplasm and nuclei was 29/55 (52.7%) (Figure 1). There was a significant correlation between survivin expression and portal vein thrombi and intrahepatic metastatic nodes ($P < 0.05$). However, it was not related to the following factors: age and sex of the patient, tumor location, tumor differentiation, tumor size, presence of tumor capsule, clinical stage, complicating liver cirrhosis, preoperative alpha fetoprotein (AFP) level, and hepatitis B surface antigen (HBsAg) (Table 1). These findings suggest that the expression of survivin may be significantly associated with metastasis.

Relationship between expression of survivin and proliferation index

Ki-67 showed as brown-yellow particles in the nuclei after

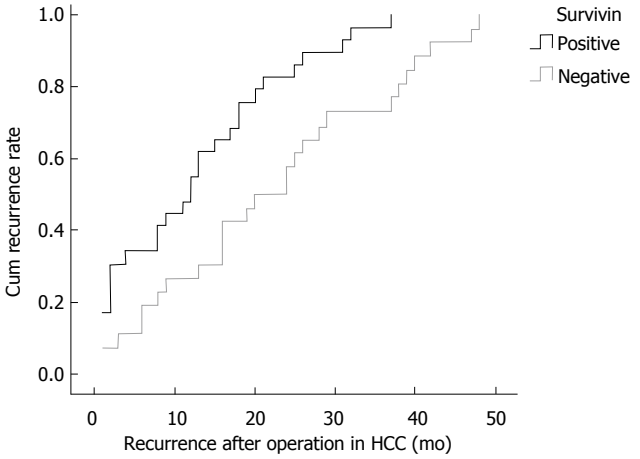

Figure 2 Correlation between survivin expression and recurrence rate of HCC after hepatectomy.

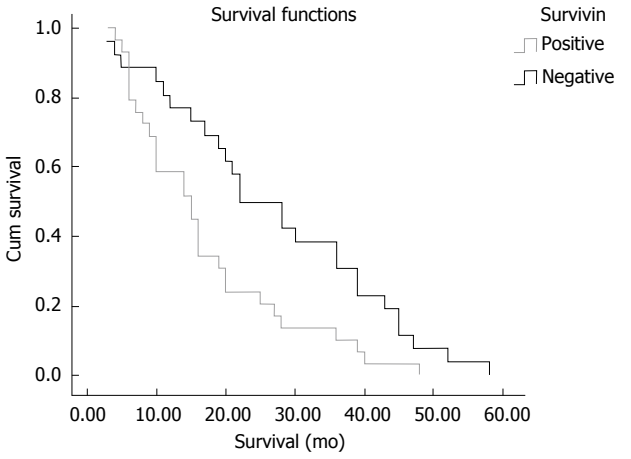

Figure 3 Kaplan-Meier curves for overall survival rates of patients with HCC according to survivin expression.

staining. Ki-67 labeling index in survivin-positive cancer was 33.83% ± 18.90%, while it was 19.60% ± 19.35% in negative tumor. The difference was significantly different ($P < 0.05$). This suggests that the expression of survivin may promote the proliferation of HCC.

Relationship between expression of survivin and recurrence and prognosis of HCC

The 1- and 3-year recurrence rates in survivin-positive HCC were 55.17% and 96.55%, respectively, while the rates were 26.91% and 73.08%, respectively, in survivin-negative HCC after hepatectomy. The recurrent time of survivin-positive HCC was significantly advanced ($P < 0.05$, Figure 2). Furthermore, the 1- and 3-year survival rates in survivin-positive HCC were 58.62% and 10.34% after hepatectomy, respectively, but for survivin-negative HCC, the rates were 76.92% and 30.71%, respectively. The 1- and 3-year survival rates were significantly lower in patients with survivin-positive HCC than those in survivin-negative HCC ($P < 0.05$, Figure 3). The expression of survivin may be used as an indicator for prognosis of HCC.

DISCUSSION

Among the recently described IAP family, survivin is characterized by a unique structure with a single BIR and no zinc-binding domain[11], and is undetectable in terminally differentiated adult tissues, but becomes notably expressed in the most common human cancers, including esophageal, stomach, colorectal, breast and pancreatic carcinoma[12-16]. Survivin has also been implicated in the control of cell-cycle kinetics and inhibition of apoptosis[17-19].

In our current study, we demonstrated that the expression of survivin was detected in 52.7% of patients with HCC, mainly localized in the cytoplasm of the carcinoma cells, with rare appearance in the nucleus. On the other hand, there have also been reports of a nuclear presence of survivin in HCC[20,21]. In previous studies, immunohistochemical analysis or RT-PCR of surgically resected tissues has revealed that approximately 30%-90% of HCC are positive for survivin expression[22-26]. The reasons for the difference may be the following: during the cell-division cycle, mRNA expression for survivin is extremely low in the G1 phase, and in the S phase is six times higher, while in the G2/M phase, the expression level of survivin increases suddenly to be 40 times higher than that in G1[27]. Therefore, the tumor cells in the G1/S phase may represent negative expression, which would lead to different expression rate of survivin in different study. On the other hand, different criteria for positive expression of survivin or experimental methods may explain the different expression rates of survivin.

Ki-67 is considered to be more useful for the determination of the proliferative activity of HCC, and is known as a risk factor for HCC. In our study, we surprisingly found that expression of the proliferation index in survivin-positive HCC was higher than that in survivin-negative HCC. The results suggest that survivin may promote cell proliferation, and contribute to the development of HCC. Survivin may interact with the cell-cycle regulator Cdk4, which leads to Cdk2/cyclin E activation and Rb phosphorylation. As a result of survivin/Cdk4 complex formation, p21 is released from its complex with Cdk4 and interacts with mitochondrial procaspase-3 to suppress Fas-mediated cell death. Survivin can also inhibit the activity of caspase-3/7 directly or indirectly, and this results in the generation and development of HCC[28-30]. The up-regulated expression of the proliferation index in survivin-positive HCC also suggests that survivin plays an important role in tumor progression.

The survivin expression in HCC was significantly correlated with portal vein thrombi and intrahepatic metastatic nodes. Therefore, survivin may play an important role in the development of HCC. Compared to survivin-negative HCC, survivin-positive HCC had a higher recurrent rate and lower 1- and 3-year survival rates. Survivin expression may play a role in the tumor biological characteristics of HCC, and may be a prognostic factor after hepatectomy. The study by Ikeguchi *et al* has also shown that high expression of survivin is associated with high recurrence and low survival rates[23]. Normal shedding of cells initiates the apoptosis process, but the over-expression of survivin exerts an anti-apoptotic effect,

which leads to a high rate of cell proliferation. Therefore, survivin may play an important role in the progression of HCC and may facilitate metastatic spread *via* the blood stream.

In conclusion, survivin expression in HCC was significantly correlated with portal vein thrombi and intrahepatic metastatic nodes. There was a significant positive correlation between survivin expression and proliferation index. Survivin plays an important role in HCC progression through promoting cell proliferation, and may be a prognostic marker for HCC.

COMMENTS

Background

Survivin is a recently described member of the family of inhibitor of apoptosis proteins (IAPs). It has been shown that survivin is strongly associated with apoptosis, cell proliferation and cell-cycle control, and becomes markedly expressed in the most common human cancers.

Research frontiers

Immunohistochemical staining of survivin and Ki-67 was performed by the standard streptavidin-peroxidase (SP) technique for paraffin sections of hepatocellular carcinoma (HCC) tissues.

Innovations and breakthroughs

We demonstrated that the positive rate of survivin in HCC was 52.7%, and a significant correlation was found between survivin expression and portal vein thrombi and intrahepatic metastatic nodes. The recurrence rate in survivin-positive HCC was significantly higher than that in survivin-negative HCC after hepatectomy. The 1- and 3-year survival rates of patients with survivin- positive tumors were significantly lower than those in patients with survivin-negative tumors. The proliferation index (Ki-67) in survivin-positive HCC was significantly higher than that in survivin-negative HCC.

Applications

Survivin may play an important role in progression of HCC by promoting cell proliferation, and may be positively correlated with a high risk of disease recurrence and poor prognosis in HCC. Its expression can serve as a prognostic factor for patients with HCC after hepatectomy.

Peer review

This is an interesting correlative study of survivin expression and survival in a cohort of 55 patients. A significantly worse survival was observed in surviving-positive tumors. The data are of high quality.

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RAPID COMMUNICATION

Relationship between vascular invasion and microvessel density and micrometastasis

Yan-Dong Wang, Pei Wu, Jia-Ding Mao, He Huang, Fan Zhang

Yan-Dong Wang, Department of Surgery, Wannan Medical College, Wuhu 241001, Anhui Province, China
Pei Wu, Jia-Ding Mao, He Huang, Department of Surgery, Affiliated Yijishan Hospital, Wannan Medical College, Wuhu 241001, Anhui Province, China
Fan Zhang, Department of Pathology, Affiliated Yijishan Hospital, Wannan Medical College, Wuhu 241001, Anhui Province, China
Correspondence to: Pei Wu, Department of Surgery, Affiliated Yijishan Hospital, Wannan Medical College, Wuhu 241001, Anhui Province, China. wp5708@sohu.com
Telephone: +86-13637134296
Received: April 17, 2007 Revised: September 12, 2007

Key words: Vascular invasion; Reverse transcriptase-polymerase chain reaction; Microvessel density; Micrometastasis

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Abstract

AIM: To evaluate the relationship between vascular invasion and microvessel density (MVD) of tissue and micrometastasis in blood.

METHODS: Vascular invasion was detected by both hematoxylin and eosin staining and immunohistochemiscal staining. Blood samples were collected from 17 patients with vascular invasion and 29 patients without vascular invasion and examined for cytokeratin20 (CK20) expression by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Microvessel density of tissue samples was also determined by immunohistochemistry using antibodies to CD105.

RESULTS: CK20 was detected in 12 of the 17 patients with vascular invasion and in 9 of the 29 patients without vascular invasion. Positive RT-PCR was significantly correlated with vascular invasion (70.6% *vs* 30.0%, $P < 0.05$). The average MVD was significantly higher in patients with positive vascular invasion than in patients with negative vascular invasion (29.2 ± 3.3 *vs* 25.4 ± 4.7, $P < 0.05$). The vascular invasion detected with hematoxylin-eosin staining was less than that with immunohistochemical staining. There was a significant difference between the two staining methods (19.6% *vs* 36.9%, $P < 0.05$).

CONCLUSION: Positive CK20 RT-PCR, depth of tumor invasion, lymph node status, metastasis and MVD are significantly correlated with vascular invasion. Immunohistochemical staining is more sensitive than hematoxylin-eosin staining for detecting vascular invasion.

INTRODUCTION

Vascular invasion is one of the most important clinicopathologic characteristics of malignant tumor. Since the initial report by Brown and Warren in 1938 demonstrating an increased visceral metastasis in rectal cancer patients with vascular invasion, a number of investigators have examined the influence of vascular invasion by colorectal cancer[1].

The presence of vascular invasion which is not a consistent finding is associated with an increased incidence of lymph node and distant metastasis and a corresponding decrease in survival[1]. Since polymerase chain reaction (PCR) invented by Mullis in 1989, it has become a standard and mature laboratory technique to detect micrometastasis in patients with malignant tumor[2]. In this study, we detected cytokeratin20 (CK20) mRNA expression in portal system blood[3-5] and microvessel density (MVD) of tissue to evaluate the relationship between vascular invasion and MVD[6] of tissue and metastasis in blood.

MATERIALS AND METHODS

Blood and tissue samples

Portal system blood was obtained before operation from 27 gastric cancer patients and 19 colorectal cancer patients. A venous catheter was inserted into the right gastric omental veins of gastric cancer patients and corresponding veins of colorectal cancer patients and blood samples were collected. The initial 5 mL blood was discarded to reduce possible contamination and the following 5 mL of blood drawn using a new syringe, was used for RNA extraction[7].

Tissue samples from 27 gastric cancer patients and

Table 1 Oligonucleotide primers

cDNA	Primer	Sequence	Product length (bp)
CK20	Outer sense	5'-GAGGTTCAAC TAACGGAGCT-3'	253
	Outer antisense	5'-TCTCTCTTCCA GGGTGCTTA-3'	
	Inner sense	5'-GCCTTGAGATA GAACTCCAG-3'	
	Inner antisense	5'-ACGTCTTCTCC TTCCAGAAG-3'	
GAPDH	Sense	5'-CAGGGCTGCTT TTAACTCTG-3'	385
	Antisense	5'-CTGTTGTCGGAG TTCTAGTAG-3'	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

19 colorectal cancer patients were formalin-fixed and parafin-embedded. The tissue samples were cut into 1 μm-thick sections, mounted onto slides coated with polylysin and examined with hematoxylin-eosin and immunohistochemical staining.

Detecting vascular invasion

Vascular invasion examined with hematoxylin-eosin staining was defined either by the presence of neoplastic cells with fibrin clots, erythrocytes, or both in endothelial cell-lined spaces without erythrocyte extravasation in the surrounding tissues or by the presence of neoplastic cells within the smooth muscle cell-lined spaces[8]. Vascular invasion examined with immunohistochemical staining was defined by the presence of at least one tumor cell cluster which was clearly visible in decorated vascular spaces where endothelial cells were stained brown[9]. According to the immunohistochemical staining, the fibrin clots or erythrocytes surrounding neoplastic cells should be considered. Vascular invasion was confirmed by at least one staning method.

Detecting CK20 mRNA in portal system blood

Isolation of mononuclear cells: Blood mononuclear cells (MNCs) were isolated by density gradient centrifugation through Ficoll-Hypaque, and washed twice with phosphate-buffered saline (PBS). Cell pellets were snap frozen in liquid nitrogen and stored at -80℃ until use.

RNA extraction: Total RNA was extracted from the MNC pellets with TRIzol reagent (Invitrogen Biotech, USA) according to the manufacturer's instructions.

Reverse transcriptase: An aliquot of 2 μg MNC RNA was pre-incubated with 0.5 μg of oligo(dT)$_{15}$ primer in 14 μL solution for 5 min at 70℃. After chilling on ice, 6 μL of 5-fold synthesis buffer, 25 U of RNase inhibitor, 1.5 μL of dNTPs (final concentration of 0.5 mmol/L) and 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase were added. The reaction mixture was then incubated for 60 min at 42℃. The reaction was terminated by heating at 95℃ for 5 min.

Table 2 Comparison between HE and immunohistochemical staining

	Vascular invasion		χ^2	*P*
	(+)	(-)		
HE staining	9	37	19.087	< 0.05
Immunohistochemical staining	17	29		

McNemar's test for correlated proportions, $\chi^2 = 8.003$, $P < 0.05$ *vs* immunohistochemical staining.

Polymerase chain reaction (PCR): PCR was carried out as described previously[10]. The sequences of primers used are shown in Table 1. To distinguish from contaminating genomic DNA, we selected both upstream and downstream primers at different exons. Integrity of the isolated RNA was demonstrated by RT-PCR analysis of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products were visualized after electrophoresis with ethidium bromide staining under a UV transilluminator.

Detecting microvessel density of tissue

CD105 antigen was detected by immunohistochemistry on a separate slide using a monoclonal mouse antibody following a standard protocol. Microvessel density was assessed as previously described[11].

Statistical analysis

Statistical analysis was performed using the likelihood chi-squared analysis, Fisher's exact test or Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of vascular invasion

Vascular invasion was detected in 9 patients with hematoxylin-eosin staining and in 17 patients with immunohistochemical staining. There was a significant difference in vascular invasion detected by the two methods (Table 2, Figure 1A and B).

Relationship between vascular invasion, MVD and micrometastasis

CK20 was detected in 12 of the 17 patients with vascular invasion and in 9 of the 29 patients without vascular invasion. Positive RT-PCR was significantly correlated with vascular invasion. The average MVD was significantly higher in patients with positive vascular invasion (29.2± 3.31) than in those with no vascular invasion (Tables 3 and 4, Figure 2).

Comparison of clinicopathologic features

Clinicopathologic features such as depth of invasion, lymph node status and metastasis were associated with the presence of vascular invasion (Table 3).

DISCUSSION

Since vascular invasion first reported by Brown and

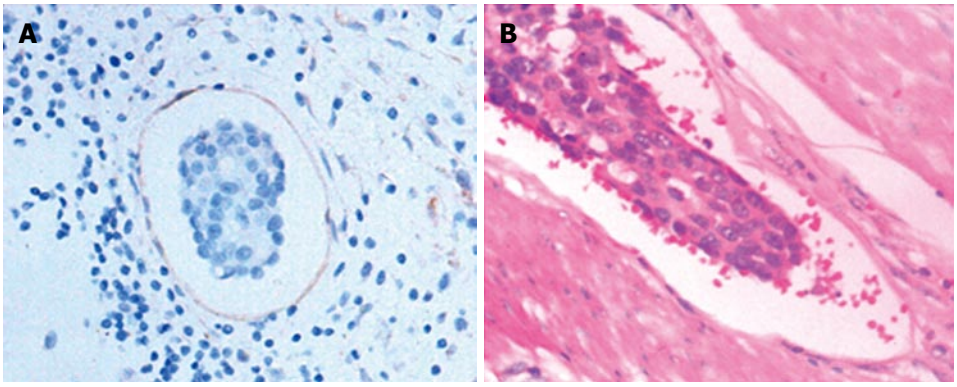

Figure 1 Immunohistochemical staining (**A**) and hematoxylin-eosin staining (**B**) of tumor cells (× 400) showing a tumor cell cluster in vascular spaces with brown-stained endothelial cells and tumor cells in blood vessel spaces with erythrocytes surrounded.

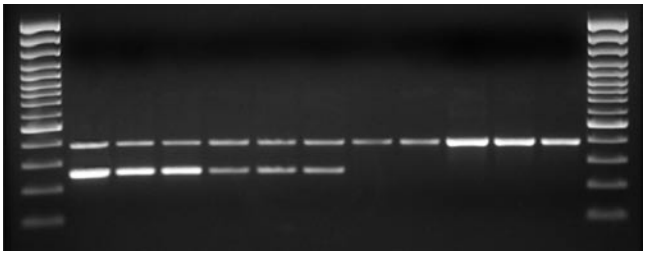

Figure 2 Expression of both CK20 mRNA and GAPDH detected in six patients and expression of only GAPDH detected in five patients.

Table 3 Comparative data on vascular invasion

	n	VI(+)	VI(-)	χ^2	*P*
CK20 mRNA					
Positive	21	12	9	6.758	< 0.05
Negative	25	5	20		
Age (yr)					
< 50	14	4	10	0.607	> 0.05
⩾ 50	32	13	19		
Size (cm)					
< 5	31	12	19	0.125	> 0.05
⩾ 5	15	5	10		
Differentiated					
Well	11	2	9	2.351	> 0.05
Moderately	20	8	12		
Poorly	15	7	8		
Serosa invasion					
Negative	14	2	12	4.440	< 0.05
Positive	32	15	17		
Lymph node metastasis					
Negative	18	3	15	5.225	< 0.05
Positive	28	14	14		
Distant metastasis					
Negative	38	9	29	16.520	< 0.05
Positive	8	8			

Statistical analysis by chi-square test. VI: Vascular invasion.

Table 4 Average number of microvessels of tissue in VI positive and negative patients

	n	MVD	*t*	*P*
VI				
Positive	17	29.2 ± 3.3	2.987	< 0.05
Negative	29	25.4 ± 4.7		

Statistical analysis of independent samples by *t* test. VI: Vascular invasion; MVD: Microvessel density.

Warren in 1938, a lot of studies have examined the influence of vascular invasion on survival[1]. Horn and colleagues found that vascular invasion is an independent prognostic factor for distant metastasis but not for survival[12]. However, Chapuis and colleagues found that vascular invasion is an independent prognostic factor for survival[13], but this was not confirmed by Wiggers *et al*[14] or Minsky *et al*[15]. In this study, we examined CK20 mRNA expression in patients with or without vascular invasion to evaluate the relationship between vascular invasion and microvessel density of tissue and micrometastasis in blood.

Vascular invasion and micrometastasis

Tumor metastasis is an orchestrated multistep process that may involve direct, hematogenous or lymphatic spread[16,17]. Tumor metastasis requires an exodus of cancer cells from the primaty site, endurance outside the hormonal and nutritional milieu of the primary site, evasion of the body' s immune surveillance, as well as adhesion, invasion, and penetration at a distant site, and organization of metastatic tissue in the secondary site with neovascularization[18]. Primary tumor invades blood and/or lymphatic vessels departing from the primary site[19]. In this study, CK20 mRNA was detected in 12 of 17 patients with positive vascular invasion, and in 9 of 29 patients with no vascular invasion, suggesting that vascular invasion is closely related to micrometastasis in blood, depth of tumor invasion, lymph node status and distant metastasis. Therefore, CK20 mRNA can be considered an indirect prognostic factor for survival. There is evidence that distant metastases are associated with the neoplastic invasion of relatively large veins at the tumor's periphery[20-22].

Vascular invasion and angiogenesis

Angiogenesis is the propelling force for tumor growth and metastasis[23-25]. To progress to a larger size, incipient neoplasms must have an angiogenic ability, which involves the sprouting of new blood vessels from preexisting capillaries, and requires the multiplication and migration of endothelial cells, remodeling of extracellular matrix, tube formation, and recruitment of surrounding structures to maintain the newly formed vessels[26]. In

this study, the average MVD was significantly higher in patients with vascular invasion than in patients with no vascular invasion, suggesting that angiogenesis is closely related with microvessel density of tissue[27] and clinical aggressiveness of tumor[28].

Detection of vascular invasion

Vascular invasion was detected with hematoxylin-eosin staining and immunohistochemical staining, respectively. The heterogeneous positive rate suggests immunohistochemical staining is more sensitive than hematoxylin and eosin staining for the detection of vascular invasion. Fibrin clots, erythrocytes, or both in endothelia-lined spaces without erythrocyte extravasation in the surrounding tissues must be concerned if detected with HE staining. However, we had to decide whether a tumor cell cluster is clearly visible in decorated vascular spaces where endothelial cells are stained brown when detected with immunohistochemical staining. Our results are consistent with the reported data[29,30].

COMMENTS

Background

Since the initial report by Brown and Warren in 1938 demonstrating an increased visceral metastasis in rectal cancer patients with vascular invasion, a number of investigators have examined the influence of vascular invasion by colorectal cancer. The presence of vascular invasion is associated with an increased incidence of lymph node and distant metastasis and a corresponding decrease in survival. However, this is not a consistent finding.

Research frontiers

Horn and colleagues found that vascular invasion is an independent prognostic factor for distant metastasis but not for survival. By multivariate analysis, Chapuis and colleagues found vascular invasion to be an independent prognostic factor for survival, but this was not confirmed by Wiggers *et al* or Minsky *et al*.

Innovations and breakthrough

Though several articles have reported the prognostic value of vascular invasion, the results are not consistent, and no study has focused on micrometastasis in patients with vascular invasion. In this study, we detected cytokeratin20 (CK20) mRNA expression in portal system blood and microvessel density of tissue to evaluate the relationship between vascular invasion and microvessel density of tissue and metastasis in blood.

Applications

We recommend vascular invasion as a method of choice for predicting prognosis of gastric and colorectal cancer patients. Patients with vascular invasion are more likely to need adjuvant therapies.

Terminology

CK20: It belongs to the epithelial subgroup of the intermediate filament family. Because of its restricted range of expression in humans, it has become an important tool for detecting and identifying metastatic cancer cells by immunohistochemistry and PCR analysis. Factor Ⅵ: Vascular invasion is usually defined by the presence of neoplastic cells with fibrin clots, erythrocytes, or both in endothelia-lined spaces without erythrocyte extravasation in the surrounding tissues or by the presence of neoplastic cells within the smooth muscle cell-lined space

Peer review

This subject is valuable for understanding the importance of vascular invasion of cancer in predicting the prognosis of such patients. It also provides a better way to increase the detection rate of vascular invasion with immunohistochemical staining.

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CASE REPORT

A phantom gallbladder on endoscopic retrograde cholangiopancreatography

Jinhong Xing, Jeremy Rochester, Caroline K Messer, Bruce P Reiter, Mark A Korsten

Jinhong Xing, Jeremy Rochester, Caroline K Messer, Bruce P Reiter, Mark A Korsten, Gastroenterology and Radiology Programs, James J. Peters VA Medical Center, Bronx, NY 10468, Montefiore Medical Center, Bronx, NY, Mount Sinai School of Medicine, New York, NY and Albert Einstein College of Medicine, Bronx, New York 10468, United States
Correspondence to: Mark A Korsten, MD, James J Peters VA Medical Center, 130 W. Kingsbridge Road, Bronx, New York 10468, United States. korsten.mark@med.va.gov
Telephone: +1-718-5849000-6753 Fax: +1-718-5791635
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Abstract

Various complications have been related to laparoscopic cholecystectomy but most occur shortly after the procedure. In this report, we present a case with very late complications in which an abscess developed within the gallbladder fossa 6 years after laparoscopic cholecystectomy. The abscess resolved after treatment with CT-guided extrahepatic aspiration. However, 4 years later, an endoscopic retrograde cholangiopancreatography (ERCP) performed for choledocholithiasis demonstrated a "gallbladder" which communicated with the common bile duct via a patent cystic duct. This unique case indicates that a cystic duct stump may communicate with the gallbladder fossa many years following cholecystectomy.

Key words: Laparoscopic cholecystectomy; Complication; Abscess; Gallbladder; Endoscopic retrograde cholangiopancreatography

Xing J, Rochester J, Messer CK, Reiter BP, Korsten MA. A phantom gallbladder on endoscopic retrograde cholangiopancreatography. *World J Gastroenterol* 2007; 13(46): 6274-6276

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INTRODUCTION

Laparoscopic cholecystectomy has been widely accepted as a preferred option for the management of patients with cholelithiasis. Complications such as biliary injuries[1,2] intestinal ischemia[3] and biliary-colonic fistula[4] have been reported, but most occur shortly after surgery. In this report, we present a case with very late complications in which an abscess developed within the gallbladder fossa 6 years after laparoscopic cholecystectomy. Four years later, despite the clinical certainty that it had been removed 10 years previously, a "gallbladder" communicating with the common bile duct *via* a cystic duct was demonstrated by endoscopic retrograde cholangiopancreatography (ERCP).

CASE REPORT

A 73-year-old male presented with complaints of right upper quadrant discomfort, fatty food intolerance and bloating. He eventually underwent an elective laparoscopic cholecystectomy in early October 1996. A J-P drainage tube was placed during the operation. The drainage was initially dark bloody, then became bilious, but gradually became clear over the next several days. The patient was discharged home after 1 wk. A follow-up abdominal CT scan was performed in May 1997, six months after the procedure. There were no fluid collections in the gallbladder fossa or peritoneal cavity.

The patient did well until early October 2002, six years after cholecystectomy, when he presented with complaints of fever, chills and mild pain in the right upper quadrant. Sonographic examination of the abdomen showed a normal sized liver with non-dilated intrahepatic ducts and common bile duct. Of note, a gall bladder-like structure was seen in the gallbladder fossa. An abdominal CT confirmed the presence of a 4 cm encapsulated fluid collection in the gallbladder fossa (Figure 1). A subsequent HIDA scan showed prompt excretion of the radiotracer into the small bowel with no evidence of biliary leak. A CT-guided transhepatic intra-abdominal aspiration was then performed (Figure 2). Turbid-appearing fluid was readily aspirated from the extrahepatic fluid collection. Gram stain of the aspirated fluids showed the presence of many white blood cells. Cultures were positive for moderate Enterobacter SP in the aspirates. The persistent low-grade fever subsided shortly after the CT-guided aspiration, and the patient was discharged home with no abdominal complaints. In August 2004 (22 mo after aspiration), an abdominal CT scan was repeated and no gallbladder fossa fluid collection was observed.

In May 2006, the patient was again admitted with the sudden onset of abdominal pain, icterus and fever. A CT scan showed a dilated common bile duct. MRCP showed intra- and extrahepatic biliary

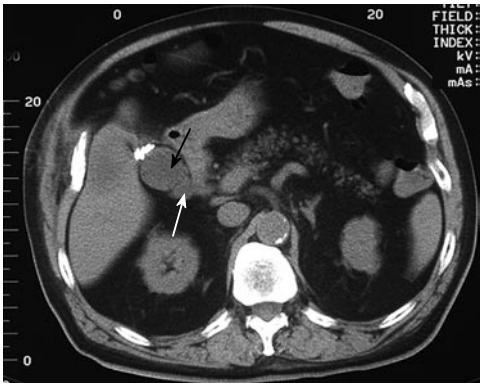

Figure 1 Abdominal CT scan six years after laparoscopic cholecystectomy. A 4 cm encapsulated fluid collection in the gallbladder fossa (black arrow) with adjacent "cystic duct-like" collections was observed (white arrow).

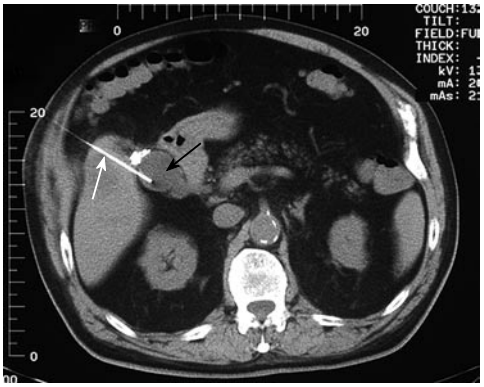

Figure 2 A CT-guided transhepatic percutaneous aspiration needle (white arrow) was seen entering the extrahepatic fluid collection (black arrow).

dilatation as well as a round, low signal filling defect within the distal common bile duct. During ERCP, the cholangiogram revealed a mildly dilated proximal common bile duct with 2 mobile stones (Figure 3A). A gallbladder-like structure that communicated with the common bile duct was also visualized in the gallbladder fossa (Figure 3B). Sphincterotomy was performed, and numerous stone fragments were removed from the CBD. A plastic biliary endoprosthesis (10F) was placed in the distal common bile duct. The patient was discharged home without any complication or discomfort two days after the ERCP. One month later, the stent was removed and the common bile duct appeared normal. A "phantom" gallbladder image reappeared on the cholangiogram.

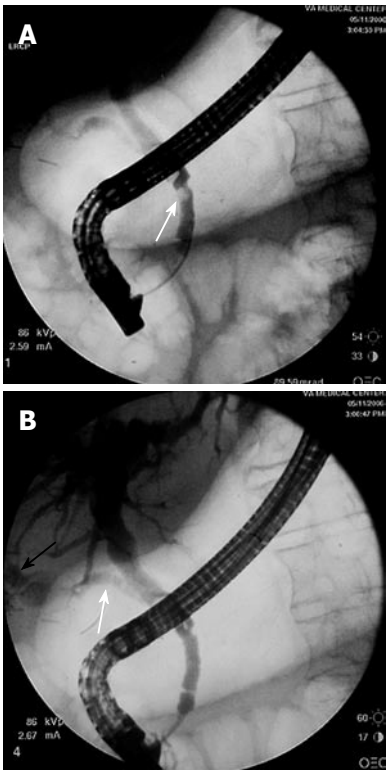

Figure 3 Calculi (white arrow) in the common bile duct were noted during the ERCP (**A**). In addition, a gallbladder-like structure (black arrow) communicated with the common bile duct *via* the cystic duct (white arrow) was also observed (**B**).

DISCUSSION

It is not uncommon to develop a fluid collection in the gallbladder fossa shortly after laparoscopic cholecystectomy. Kang *et al*[5] studied 106 consecutive patients 24 h after laparoscopic cholecystectomy using ultrasound. They identified small fluid collections in the gallbladder fossa in 56 (53.0%) patients. In another study[6], the gallbladder fossa of 70 asymptomatic patients was sonographically examined within two weeks of laparoscopic cholecystectomy. The authors reported the presence of homogeneous echogenic structures in the gallbladder bed in 35 (50%) patients, inhomogeneous structures in 25 (35.7%) cases, and, cystic structures, resembling the normal gallbladder, in 6 (8.5%) cases. The nature of the fluid collection varied, but included seromas, hematomas, abscesses and biloma.

In the present case, the fluid collection inside the gallbladder fossa observed 6 years after the procedure proved to be an abscess on the basis of the turbid, non-bilious, culture positive fluid retrieved during CT-guided aspiration. The abscess resolved after percutaneous transhepatic aspiration and antibiotic treatment. The most recent finding in this patient, a gallbladder-like structure inside the gallbladder fossa that directly communicated with the common bile duct, is likely related in some manner to this prior abscess. One possibility is that the previously documented abscess may have resolved by fistualizing into the cystic duct. Alternatively, increased pressure within the cystic duct as a result of biliary obstruction from choledocholithiasis may have caused rupture of the stump and its subsequent breakthrough into a residual cavity in the gallbladder fossa.

By whatever mechanism, this case demonstrates that, post-cholecystectomy, a cystic duct stump may potentially communicate with extrabiliary structures in and around the gallbladder fossa. In this respect, we believe that this is the first reported case in which a cystic structure resembling a radiologically typical "gallbladder" was seen on ERCP many years after this organ was surgical removed.

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CASE REPORT

Prolonged cholestasis following successful removal of common bile duct stones: Beware patients on estrogen therapy

JM Dunn, A McNair

JM Dunn, A McNair, The Queen Elizabeth Hospital, Woolwich, London, United Kingdom
Correspondence to: A McNair, Gastroenterology Department, The Queen Elizabeth Hospital, Stadium Road, Woolwich, London, SE18 4QH, United Kingdom. a.mcnair@nhs.net
Telephone: +44-8-8365579 Fax: +44-8-8365581
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Abstract

There are various well described forms of chronic cholestatic jaundice in adults, such as autoimmune cholangitis, drug-induced cholangitis and intrahepatic cholestasis of pregnancy. We present two cases of prolonged cholestasis following removal of gallstones at endoscopic retrograde cholangiopancreatography (ERCP) and subsequent clear cholangiography. Both patients were taking oral estrogens at the time of presentation, which were subsequently withdrawn. The first case responded rapidly to corticosteroid treatment, and the second case had a much slower resolution with ursodeoxycholic acid. Both cases highlighted the significance of estrogen-induced cholestasis in female patients with protracted jaundice following ERCP and removal of intra-ductal stones. After oral estrogens are discontinued, a short course of steroids needs to be considered.

Key words: Estrogen; Cholestasis; Gallstones; Steroids; Ursodeoxycholic acid

Dunn JM, McNair A. Prolonged cholestasis following successful removal of common bile duct stones: Beware patients on estrogen therapy. *World J Gastroenterol* 2007; 13(46): 6277-6280

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INTRODUCTION

Estrogens have been a well-recognized cause of cholestatic jaundice since 1962[1], and are commonly used in an experimental model of hepatocellular cholestasis. The similarity between such drug reactions and the syndrome of intrahepatic cholestasis of pregnancy has been reported[2]. Withdrawal of the estrogenic effect by delivery of the fetus or drug withdrawal leads to improvement of liver function. This tends to occur over several weeks or months[3]. It has been shown in observational studies that estrogen therapy is an important risk factor for gallbladder disease[4]. A recent randomized double-blind placebo-controlled trial of otherwise healthy postmenopausal women has demonstrated that the risk of adverse biliary tract outcomes, such as cholecystitis, is substantially increased by exogenous estrogen therapy[5]. Estrogens are thought to promote gallstone formation by significantly elevating the biliary cholesterol saturation index and a reduction of the nucleation time[6]. We present two cases of prolonged cholestasis after removal of obstructing common bile duct (CBD) stones at endoscopic retrograde cholangiopancreatography (ERCP).

CASE REPORTS

Case 1

A 25-year-old Caucasian woman presented at 6 wk post partum with pruritus and right upper quadrant pain. There was no jaundice evident, and she had been asymptomatic throughout her pregnancy. She had stopped taking Microgynon (ethinlyestradiol 30 μg and levonorgestrel 150 μg) 1 year previously. Her mother had undergone cholecystectomy aged 50 years. Physical examination was unremarkable, and liver function tests showed a total serum bilirubin level of 43 μmol/L, alanine aminotransferase (ALT) 187 IU/L, alkaline phosphatase (ALP) 81 IU/L and gamma-glutamyltranspeptidase (γ-GT) 88 IU/L (Figure 1). Liver ultrasound scan showed multiple calculi in the gall bladder and a CBD of 6 mm, with no evidence of intraductal stones.

She presented again 6 wk later with worsening pruritus, biliary colic and jaundice. The patient had restarted Microgynon in the interim period. She denied exposure to alcohol or illegal drugs. There were no risk factors for viral hepatitis. Her weight had dropped by 8 kg and she had icterus. Abdominal palpation revealed a tender right upper quadrant but no hepatosplenomegaly. Retesting of liver biochemistry showed a total serum bilirubin level of 163 μmol/L and a twofold increase in serum ALP. ALT remained elevated at 163 IU/L. Abdominal ultrasound scanning showed a thick-walled edematous gallbladder with multiple small calculi, one impacted in the neck of the gallbladder. The CBD measured 3.5 mm.

Endoscopic retrograde cholangiopancreatography (ERCP) was performed and revealed two calculi in the CBD, the largest measuring 5 mm. A 12-mm

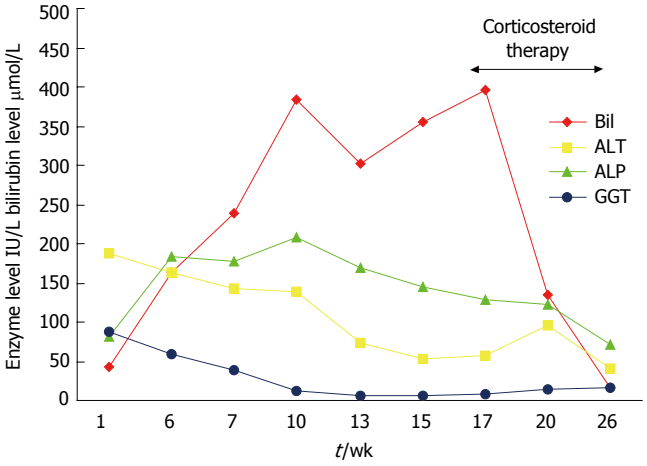

Figure 1 Case 1: Liver biochemistry and response to corticosteroids.

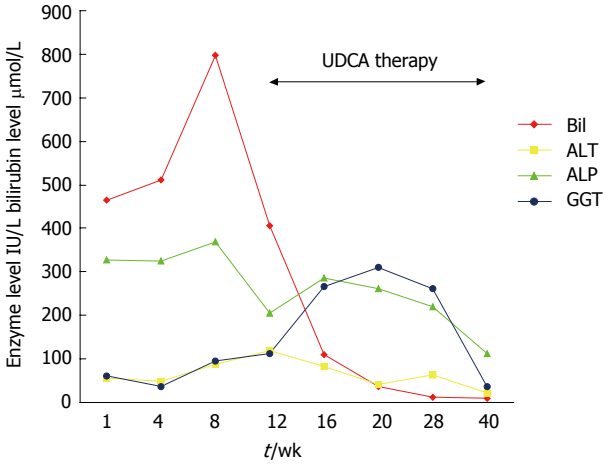

Figure 2 Case 2: Liver biochemistry and response to UDCA.

sphincterotomy was made and the CBD dredged with a balloon. Both stones were removed and an occlusion cholangiogram confirmed clearance of the duct. Her total serum bilirubin level continued to rise over the next 10 wk. A second ERCP performed 3 wk after the first showed a dilated CBD, but no obvious filling defects. The sphincterotomy was extended. A balloon dredge and occlusion cholangiogram confirmed that the duct was clear. Microgynon was stopped at this point. Autoantibody profile and hepatitis A, B and C serology were negative. Ceruloplasmin and ferritin levels were normal. The hemoglobin level was stable and the haptoglobin level normal. Because of persisting jaundice, a third ERCP was performed 3 wk later and revealed a normal condition.

Seventeen weeks following the initial presentation (7 wk after stopping the oral contraceptive pill), total serum bilirubin level continued to rise and reached 386 μmol/L. The patient was treated with prednisolone 40 mg/d, which was reduced by 5 mg/d at weekly intervals over a period of 8 wk. Response to treatment is shown in Figure 1. Her symptoms improved dramatically during treatment with the corticosteroid, with a concomitant restoration of normal liver biochemistry. She has now been followed up for more than 12 mo and is asymptomatic with weight gain of 10 kg.

Case 2

A 66-year-old Caucasian woman presented with a 4-wk history of jaundice, right upper quadrant pain and pruritus. She had a past history of hysterectomy and bilateral salpingo-oopherectomy, carried out when she was 40 years old, complicated by post-operative deep vein thrombosis. She started Premarin (conjugated estrogens) 625 μg for menopausal symptoms when she was 50 years old, and had been taking this continuously up to the time of her presentation. The patient had no history of jaundice, hepatitis, blood transfusion or travel outside Western Europe. She had no risk factors for viral hepatitis and did not drink alcohol. On examination she was jaundiced, with no signs of chronic liver disease, and was afebrile. Tenderness on palpation of the right hypochondrium was evident, and the liver was palpable 2 cm below the costal margin.

Laboratory investigation revealed a total serum bilirubin of 464 μmol/L, ALT 56 IU/L, ALP 328 IU/L and γ-GT 60 IU/L. Serological tests for hepatitis A, B and C were negative. Anti-nuclear factor, and anti-mitochondrial and smooth muscle antibodies were also negative. Ultrasound examination of the liver revealed a dilatated CBD measuring 10 mm and multiple small radio-opaque calculi in the duct.

The patient underwent ERCP and sphincterotomy; all intraductal stones were removed with a balloon. However, 8 wk after her initial presentation, the patient remained markedly jaundiced with worsening pruritus, malaise, anorexia and weight loss of 6 kg. Liver function tests had worsened: total serum bilirubin 798 μmol/L, ALT 87 IU/L, ALP 370 IU/L and γ-GT 96 IU/L (Figure 2). Premarin was stopped.

A CT scan showed a normal gallbladder, with no evidence of malignancy and no biliary tree dilatation. A repeat ERCP showed a normal biliary system with no evidence of retained CBD stones. Ursodeoxycholic acid (UDCA) 1200 mg/d was commenced following ERCP. Four weeks later, her symptoms had begun to improve, and her bilirubin level had fallen to around half its peak value. After a further month, her symptoms had resolved completely, although her liver biochemistry did not completely return to normal until 40 wk after her initial presentation.

DISCUSSION

In both cases, prolonged cholestasis after removal of CBD stones at ERCP was thought to have been caused by oral estrogen therapy. The association between oral estrogen-containing preparations and several cholestatic syndromes has been well recognised since 1962[1]; and of the canalicular type with little portal inflammation. It is thought that these reactions are because of the estrogen component; hence it is widely used as an experimental model of hepatocellular cholestasis. This effect is mediated by its glucuronidated metabolites, which inhibit canalicular bile salt and glutathione excretion, which results in inhibition of bile salt transport[7].

Clinical features include malaise, pruritus, jaundice and

anorexia with subsequent weight loss, were seen in both our patients. These features are also seen in the clinical syndrome of intrahepatic cholestais of pregnancy (ICP). Indeed a Chilean group has found that, in 42 patients with cholestatic jaundice following use of oral estrogen compounds, 27 had previously jaundice and pruritus during pregnancy, which suggests a link between the two disorders[2]. This must be considered in the context of the strong geographical predilection of this disease to Chile. Crucially, withdrawal of the estrogenic effect by delivery of the fetus or drug withdrawal leads to improvement in liver function. This tends to take place over several weeks or months. Neither of the patients described here had a history of ICP, although liver function tests had not previously been performed.

Case 1 had proven gallstones at 6 wk post partum and liver function tests were consistent with stones in the CBD. The rapid deterioration in liver function both clinically and biochemically after commencing oral estrogens, and the resolution on discontinuation of the treatment support the hypothesis that the cholestasis was a drug-induced liver injury. Other potential causes were excluded. In particular, the absence of antimitochondrial antibodies, normal intrahepatic bile ducts on ERCP, and the spontaneous normalization of liver biochemistry excluded both primary biliary cirrhosis and primary sclerosing cholangitis.

Case 2 had been on estrogen replacement therapy for 16 year at the time of her presentation with obstructive jaundice. However, the only factor that could account for the prolonged cholestasis was the estrogen therapy, and the temporal relationship between stopping estrogens and improvement in liver function further supports the conjecture that hormone replacement therapy was responsible (although UDCA therapy may have played a role in the resolution of the cholestasis).

Since estrogen therapy alone did not cause jaundice in this patient, there must have been an interaction between the effects of mechanical obstruction to bile flow and the cholestatic effects of estrogens. The patient has remained off estrogens since the procedure.

The cause of the differences in time for resolution of jaundice in cases 1 and 2 is unclear. The jaundice in both cases was likely to have resolved following the withdrawal of estrogens, and it is possible that the longer duration of jaundice in case 2 was simply a reflection of the variable length of time for estrogen-induced cholestasis to resolve following treatment withdrawal. Typically, this takes several weeks to months, but it has been reported to take up to 10 years[3]. It is also possible that the difference in time for the jaundice to resolve reflected differences in the modes of action of the drugs used to treat the cholestasis. The anti-inflammatory effects of corticosteroids may be of benefit for individuals with a pre-existing mechanical obstruction. It is known that when biliary obstruction occurs, an inflammatory response is mounted with the release of pro-inflammatory cytokines (e.g. tumor necrosis factor-α and interleukin-1), a reduction in the expression of nuclear bile acid receptor, and infiltration of neutrophils, all of which are thought to aggravate cholestatic injury[8].

Corticosteroid use has previously been described in two cases reports of prolonged cholestasis following ERCP and successful removal of gallstones[9]. The patients were not taking estrogens (both were male) and the authors hypothesized that the canalicular function had been directly compromised by a mechanical obstruction. They also postulated that the radiocontrast medium infused under high pressure during ERCP may have had a toxic effect, with disruption of the canalicular membrane, but no evidence was given to support this theory. The two cases presented differed from both our patients, who were taking oral estrogens. Whilst the onset of jaundice appears to have been precipitated by the presence of obstructing intraductal calculi, the estrogen therapy appears to have been responsible for the persistence of cholestasis. Presumably, the biliary obstruction in some way sensitized the biliary canaliculi to the cholestatic effects of estrogens, perhaps through an associated inflammatory response (discussed above) that led to the prolonged cholestasis. The interaction between these two factors may in part be idiosyncratic and/or dependent on genetic factors, since this has not previously been reported.

UDCA was used in the second case because of concerns about side effects, particularly steroid-induced osteoporosis in this postmenopausal woman. Its use resulted in a less impressive response, but it may not have influenced the course of the cholestasis. However, evidence from a number of large studies has confirmed the efficacy of UDCA in ICP, and the drug is now used routinely for treating this condition[10]. The use of UDCA in treating cholestasis secondary to oral estrogen therapy in humans has not been reported, although ethinylestradiol-induced cholestasis in rats has been shown to respond to UDCA[11]. The authors of that study concluded that UDCA increased bile flow by increasing bile acid secretion, through the normalization of the expression of the canalicular bile salt export pump. UDCA has also been noted to decrease the glucuronidation of estrogens, thereby decreasing the production of cholestatic metabolites[12].

Although corticosteroid treatment appeared more effective than UDCA, the two agents have been compared in a randomized study in ICP. Treatment with UDCA led to a significant reduction in ALT and bilirubin and an improvement in pruritus, while dexamethasone had no such effects[13].

Endoscopists who carry out ERCPs should be aware of the potential causes of persistent jaundice following the removal of intraductal stones. In female patients with protracted jaundice, the possibility of estrogen-induced cholestasis should always be considered, and oral estrogens discontinued. In such cases, a short course of corticosteroid treatment should be considered.

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CASE REPORT

Penetrating ectopic peptic ulcer in the absence of Meckel's diverticulum ultimately presenting as small bowel obstruction

Hilary Hurley, Ronan A Cahill, Paul Ryan, Ashraf I Morcos, Henry P Redmond, Helen M Kiely

Hilary Hurley, Ronan A Cahill, Henry P Redmond, Department of Surgery, Cork University Hospital, Cork, Ireland
Paul Ryan, Helen M Kiely, Department of Pathology, Cork University Hospital, Cork, Ireland
Ashraf I Morcos, Department of Gastroenterology, Mercy University Hospital, Cork, Ireland
Correspondence to: HP Redmond, Professor, Department of Surgery, Cork University Hospital, Wilton, Cork, Ireland. rcahill@rcsi.ie
Telephone: +353-21-4922371
Received: July 16, 2007 Revised: September 6, 2007

Abstract

We report here how a heterotopic penetrating peptic ulcer progressed to cause small bowel obstruction in a patient with multiple previous negative investigations. The clinical presentation, radiographic features and pathological findings of this case are described, along with the salient lessons learnt. The added value of wireless capsule endoscopy (WCE) in such circumstances is debated.

Key words: Non-Meckelean ectopic peptic ulcer; Bowel obstruction

Hurley H, Cahill RA, Ryan P, Morcos AI, Redmond HP, Kiely HM. Penetrating ectopic peptic ulcer in the absence of Meckel's diverticulum ultimately presenting as small bowel obstruction. *World J Gastroenterol* 2007; 13(46): 6281-6283

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INTRODUCTION

Heterotopic gastric mucosa resulting in peptic ulceration in the absence of a Meckel's diverticulum is a most unusual cause of small bowel symptomatology[1]. Although wireless capsule endoscopy (WCE) enables painless visualization of the small bowel in a non-invasive manner, its added value in cases not presenting with occult gastrointestinal blood loss is unclear. In this report, we consider what extra value this technology added to the care of a patient who had had multiple negative standard investigations, and ultimately required a laparotomy and ileal resection for definitive management.

CASE REPORT

A 77-year-old man presented to the Accident and Emergency Department of our hospital with subacute small bowel obstruction, having complained for many years of intermittent severe central abdominal discomfort associated with episodic distension and constipation. Prior to this admission, these symptoms had been extensively investigated by means of plain radiology, computerized tomography (CT) and barium contrast studies, as well as by both upper and lower gastrointestinal endoscopy, but to no avail. Furthermore, laparotomy at the time of one such presentation had been performed but without therapeutic benefit, as no evidence of overt intestinal pathology or abnormality was found (in particular, there was no evidence of Meckel's diverticulum). On this latest presentation, the clinical examination was consistent with subacute small bowel obstruction (distended but soft, moderately tender abdomen, with hyperactive bowel sounds and signs of extracellular fluid depletion). Plain abdominal radiography revealed the presence of dilated loops of the small bowel, while subsequent abdominal CT confirmed this finding but did not identify a transition point or any mass lesion. Initial treatment involved correction of fluid and electrolyte abnormalities and nasogastric aspiration for symptomatic relief. Mindful of his past history, when the symptoms persisted for more than 48 h, small bowel barium follow-through was performed. This also failed to define any specific pathology, although the barium failed to progress beyond the proximal jejunum.

As the patients symptoms had somewhat relented by this stage and he resumed passing flatus again, he was therefore permitted some oral diet which he tolerated despite some intermittent, colicky pain. Although a second laparotomy was advocated because of the ongoing persistence of low-grade symptoms, the patient was reluctant, on empiric reasons alone, to consider this given the lack of impact from his previous operation. WCE was therefore performed on a somewhat speculative basis. This test again demonstrated a prolonged transit time through the small bowel (the capsule had not progressed through the jejunum after 7 h), but the last frame of the study showed a raised mucosal jejunal lesion adjacent to an area of severe luminal stenosis (Figure 1). Along with the non-resolving symptoms, identification of this suspicious mucosal lesion, compounded by capsule retention, strengthened the case for exploratory laparotomy. The patient consented. At operation, two

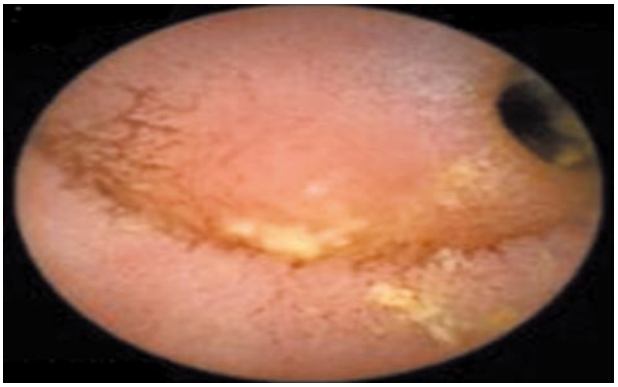

Figure 1 Image of jejeunal luminal stenosis from WCE.

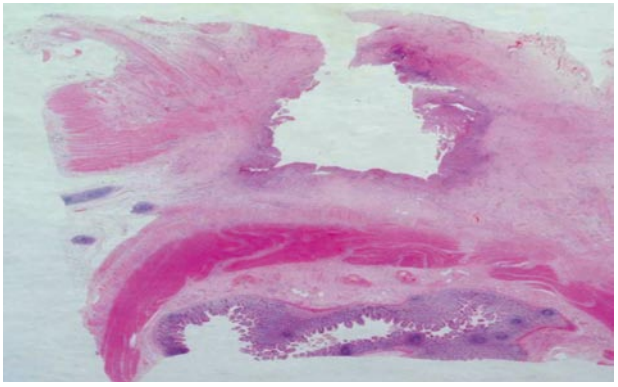

Figure 2 Photomicrograph of a deep penetrating ulcer with underlying fibrosis replacing muscularis propria, extending through the serosa adherent to an underlying small bowel loop (lower field).

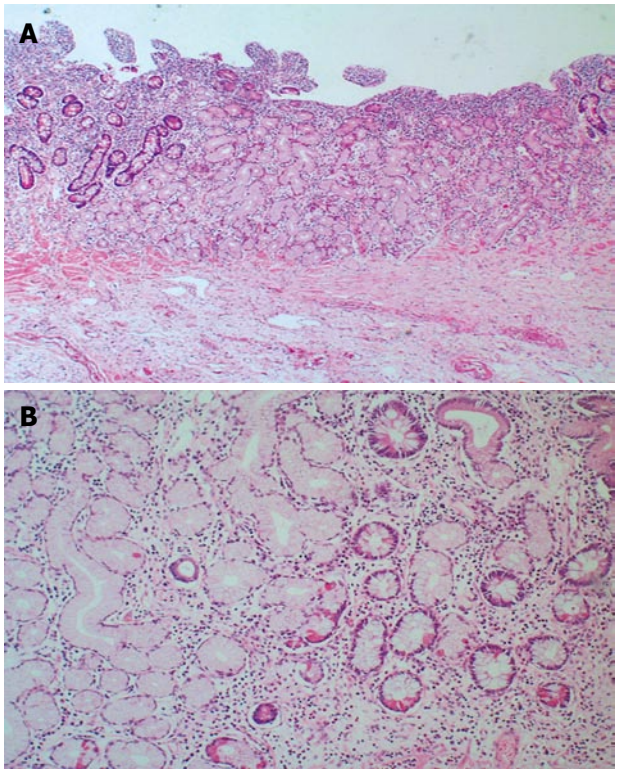

Figure 3 Photomicrographs showing (**A**) heterotopic gastric foveolar mucosa with a few normal small intestinal glands; and (**B**) scattered cells with eosinophilic cytoplasm compatible with acid-secreting cells.

adjacent loops of densely adherent jejenum were obvious among other areas of peritoneal adhesions. There were also features of chronic dilatation proximal to an area of luminal stricturing within one of these loops of bowel. Careful adhesiolysis permitted resection of a segment of chronically obstructed aperistaltic small bowel, as well as allowing for the retrieval of the capsule. A primary hand-sewn end-to-end jejunojejunal anastomosis was performed. Opening of the specimen subsequently revealed an underlying oval mucosal ulcer with punched out edges extending through the wall, which caused fibrosis and adhesion to the adjacent loops of bowel (Figure 2). Histological examination of the resected specimen identified foci of heterotopic gastric mucosa adjacent to this deep penetrating chronic peptic ulcer (Figure 3). The patient thereafter made an uncomplicated recovery and remains symptom-free 6 mo later.

DISCUSSION

Heterotopic gastric mucosa in the small bowel, other than in Meckel's diverticulum or other congenitally anomalous bowel, is rare[2]. Such lesions usually cause small bowel obstruction, either secondary to a mechanical lesion in the lumen[1], or intermittently due to intussusception of the ectopic mass[2-5]. Acute presentation with a perforating ulcer is rarely seen; indeed, there have been only five cases reported in the literature[1-4], with a further five being reported as causing hemorrhage due to ulcer penetration[1]. In our patient, an ulcerating ectopic mucosal lesion presented unusually as small bowel obstruction secondary to an extensive fibrotic reaction. In this case, there was no macroscopic evidence of diverticulum, luminal duplication, or congenital anomaly. Due to the dense adhesional mass and desmoplastic reaction, it was not possible to identify whether the ulcer was on the mesenteric or antimesenteric border of the small bowel, but the penetrating jejunal ulcer was clearly present at the site of the transition point between the dilated and normal caliber small bowel. Microscopic examination of the lesion yielded evidence of ectopic gastric mucosa adjacent to the deep penetrating ulcer, associated with features of chronic inflammation and fibrosis. A diagnosis of chronic peptic ulcer, secondary to heterotopic gastric mucosa with reactive adjacent fibrosis and adhesions was therefore concluded.

The development of WCE technology has changed investigative endoscopy of the small bowel into a much less invasive and more complete examination. A now-proven reliable tool for verifying the state of the small bowel[2], it has been particularly successful in finding the cause of obscure gastrointestinal bleeding[3] and chronic diarrhea, and in evaluating the extent of Crohn's disease. It is not generally used in cases of small bowel obstruction, due to the risk of capsule retention that should precipitate surgical intervention. In patients with undiagnosed abdominal pain, the yield from the use of WCE appears low, whereas in this case, it proved useful in securing a pathological diagnosis and furthering management[4], as there was an

understandable reluctance by the patient to proceed to a second laparotomy without a strong positive indication and likelihood of therapeutic benefit. This case therefore adds some weight to the argument that judicicious use of WCE in small bowel obstruction may identify the site of obstruction and guide surgical intervention[5,6]. In particular, this could be of great significance for a surgeon attempting to localize otherwise non-apparent pathology intraoperatively. In this case, WCE was considered only in the setting of multiple negative investigations and a high probability of operative intervention, given the high risk of capsule retention.

While WCE added little objective evidence to the findings at the latter operation, it is intriguing to speculate how this test may have guided intervention at the time of first surgery. Although small bowel ulcers, ileal tuberculosis and even worm infestation have previously been demonstrated by WCE, most small bowel pathology found to date has been Crohn's disease or NSAID-related lesions of the distal small bowel[5]. There is only one previous case in the literature of heterotopic gastric mucosa of the small bowel identified by capsule endoscopy[6]. Although the captured images in this case did not diagnose the specific lesion, they did suggest a mucosal anomaly and secondary stenosis, which was subsequently histologically identified as ulcerating heterotopic gastric mucosa. If knowledge of such an abnormality had been available prior to the first operation, perhaps that intervention could have been tailored with therapeutic benefit.

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LETTERS TO THE EDITOR

Hepatic hydrothorax occurring rapidly after manual abdominal compression

Kazufumi Dohmen, Hirofumi Tanaka, Masatora Haruno, Yoshiyuki Niho

Kazufumi Dohmen, Hirofumi Tanaka, Masatora Haruno, Yoshiyuki Niho, Internal Medicine, Chihaya Hospital, Fukuoka 813-8501, Japan
Correspondence to: Dr. Kazufumi Dohmen, Internal Medicine, Chihaya Hospital, 2-30-1 Chihaya Higashi-ku, Fukuoka 813-8501, Japan. dohmen-kzfm@cool.odn.ne.jp
Telephone: +81-92-6612211 Fax: +81-92-6830411
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Abstract

Hepatic hydrothorax is a relatively infrequent but potentially serious complication of liver cirrhosis that often causes respiratory dysfunction. Several hypotheses for the development of hepatic hydrothorax have been suggested to explain a transdiaphragmatic shift of ascitic fluid through small defects between the peritoneal cavity and the pleural space. However, the rapid development of hydrothorax within several hours is seldom encountered. In addition, the causal factors for rapid passage of ascitic fluid into the pleural cavity are unknown. This report describes a patient with liver cirrhosis who suffered rapid development of a hydrothorax after manual compression of the abdomen.

Key words: Hydrothorax; Liver cirrhosis; Abdominal compression

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TO THE EDITOR

A 63-year-old female with Child-Pugh grade C liver cirrhosis was admitted to Chihaya Hospital, Fukuoka, Japan because of ascitic fluid and hepatic encephalopathy. Physical examination revealed a markedly distended abdomen and peripheral edema. Her laboratory blood tests showed a white blood cell count of 9550/L, hemoglobin of 7.6 mg/dL, platelet count of 54000/L, aspartate aminotransferase 39 IU/L (normal 7-38), alanine aminotransferase 16 IU/L (normal 4-43), gamma glutamyltransferase 45 IU/L (normal 16-73), alkaline phosphatase 600 IU/L (normal 104-338), serum albumin 2.0 mg/dL (normal 3.8-5.2), total bilirubin 1.5 mg/dL (normal 0.2-1.1), and serum ammonia 255 μg/dL (normal 30-86). Chest radiography showed no pleural effusion (Figure 1), while abdominal computed tomography imaging was characteristic of cirrhosis with a massive amount of ascitic fluid (Figure 2).

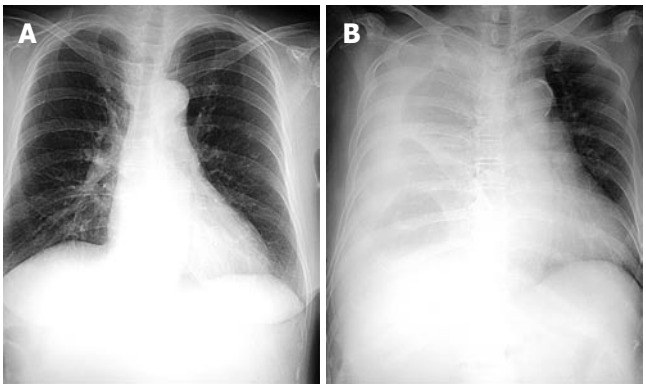

Figure 1 Chest X-ray. **A**: Before manual compression of the abdomen; **B**: on the next day, after manual compression of the abdomen.

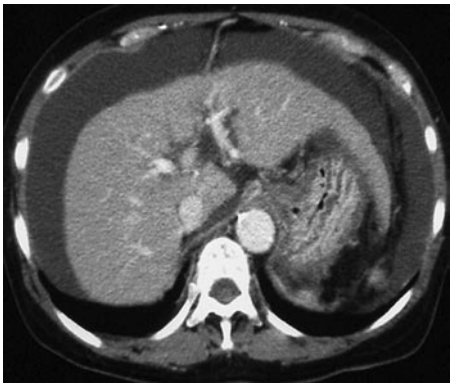

Figure 2 Abdominal computed tomography before manual compression of the abdomen shows liver cirrhosis with a massive volume of ascitic fluid.

After admission, the patient was treated with branched-chain amino acid supplementation, diuretics and lactulose enema, in combination with antibiotics for ascites and hepatic encephalopathy. Hepatic encephalopathy had been well controlled; however, it manifested in association with constipation on fourteenth day after admission. Constipation was not relieved after the administration of laxative drugs and a lactulose enema. Therefore,

manual abdominal compression was performed gently for approximately 1 h by a nurse to accelerate bowel movement. After that, a large amount of stool was evacuated and hepatic encephalopathy improved. However, during the following night, progressive dyspnea occurred and analysis of blood oxygen showed hypoxemia. Chest radiography on the next day revealed a massive right-sided pleural effusion (Figure 1), while ultrasonography showed that the ascitic fluid had disappeared. Pleural effusion obtained by thoracenteses showed a transudate, which indicated the rapid migration of ascitic fluid into the right hemithorax.

The transfer of large volumes of fluid from the abdomen to the pleural space through defects in the diaphragm can easily be understood. Once the diaphragmatic communications begin to leak fluid in response to abdominal pressure such as a cough or muscle strain, the combination of a hydrostatic gradient of negative intrathoracic pressure and positive intra-abdominal pressure passively produces a unidirectional flow of ascitic fluid into the pleural space[1,2]. The occurrence of hydrothorax induced by tense and prolonged abdominal compression has not previously been reported, although there have been a few reports demonstrating the actual condition of the rapid occurrence of hepatic hydrothorax[3].

Interestingly, in this case with cirrhosis and a massive volume of ascitic fluid, the imaging data and clinical course strongly suggested that the rapid migration of ascitic fluid into the pleural space occurred due to the manual abdominal compression. Therefore, clinicians need to be aware of the possibility that massaging the abdomen can cause a hepatic hydrothorax in patients with a massive volume of ascitic fluid.

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Department of Medicine Schlosspark-Klinik, Humboldt University, Heubnerweg 2, Berlin D-14059, Germany

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symposia@falkfoundation.de

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Banff-AB
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Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
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Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
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www.bsg.org.uk

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11-15 April 2007
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easl2007@easl.ch
www.easl.ch/liver-meeting

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18-22 April 2007
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Meeting Falk Symposium 159: IBD 2007-Achievements in Research and Clinical Practice
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Patent (list all authors)

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Statistical data

Present as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as γ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2 not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyr*A, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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EDITORIAL

What's wrong with sentinel node mapping in colon cancer?

Ronan A Cahill

Ronan A Cahill, Department of General Surgery, Cork University Hospital, Wilton, Cork, Ireland
Correspondence to: Ronan A Cahill, IRCAD/EITS, 1 Place de l'Hopital, Strasbourg 67091, France. rcahill@rcsi.ie
Telephone: +353-87-2886417
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Abstract

Despite near-universal embrace of the concept and clinical relevance of lymphatic mapping for sentinel node identification and analysis for cancers of the breast and integument, the same technique has struggled to a find a role in gastrointestinal cancers in general and, perhaps, in colon cancer in particular. Despite many studies demonstrating its feasibility in malignancies of the large bowel, concern is continually aroused by the variable and often unacceptably low sensitivity rates. Additionally, many confess uncertainty as to what benefit it could ever confer to patients even if it were proven sufficiently accurate given that standard surgical resection incorporates mesenteric resection anyway. However, the huge impact sentinel node mapping has had on clinical practice in certain cancers means that each of these aspects merit careful reconsideration, from very first principles.

Key words: Sentinel node; Lymphatic mapping; Colon cancer

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Despite being initially proposed many years before, the sentinel node concept has only recently impacted upon clinical practice. The major landmark work in validating the theory took place in melanoma patients[1], but the proof of the concept was quickly (and relatively painlessly) transferred to breast cancer[2]. However, perhaps the most salient aspect evident on reviewing these seminal publications now is their consistent focus on confining the technique to relatively early stage cancers. The rationale underlying this is that larger tumours involve a greater area of lymphatic channels and also that more advanced tumours may demonstrate aberrant lymphatic dynamics, both within the primary and in the lymphatic channels in the immediate vicinity. Lymphatic mapping is, therefore, currently recommended only for intermediate thickness melanoma (1.2 mm to 3.5 mm deep)[3] and T1 and 'early' T2 breast tumours[4,5]. As discussants of the topic with regard to colon cancer largely tend to overlook this basic tenet, it is worth reviewing the literature for *in vivo* sentinel node detection in colon cancer from this perspective (*ex vivo* work has been excluded from this review as it primarily is of interest from the point of view of pathological evaluation rather than surgical approach).

To date, there have so far been 37 publications in the English language describing over 2500 patients (although some studies may have overlapped their patient groups). Explicit cognisance of tumour advancement and mural penetration has mostly only been peripherally addressed in these studies, however, and tumour size hardly at all. Only four studies excluded patients with evident macroscopic lymphadenopathy[6-9] and it is interesting to note that each of these despite being relatively small studies from groups outside of the main proponent centres have excellent results given the other characteristics of their patient cohorts (see below). The authors with the most consistently impressive results have also tended to include relatively high proportions of "early tumours" (approximately 50% or more of their cancers have been T1 or T2)[2,5,10]. However, when collaborating in a multicentre study resulting in patients with tumours of somewhat more advanced T-stage, sensitivity rates reduced. Further examination of these patients led to the conclusion that this was primarily due to lymphatic obstruction by tumour[11]. Furthermore, when a related group described a cohort of somewhat more advanced primary tumours (at least in terms of T-stage) for the purposes of a different study their lymphatic mapping results also appeared worse (82% detection rate with 12% false negative rate) than those previously published[12]. Other authors have also reported high sensitivity rates when dealing with earlier tumours although this aspect of the patient demographic was not specifically teased out[13,14].

Conversely, the studies with the highest false negative rates have also tended to have the greatest proportion of T3/T4 tumours in their study cohorts (with the T3-T4 proportion representing at least two thirds of the total study group)[15-21]. Better results in such patient populations is reported in those studies which specifically excluded those with evident lymph node disease intraoperatively[9]. Of the studies that have considered any potential impact of T-stage, Viehl *et al*[22] found identification rates most significantly affected by tumour size:dye instillate ratio, Saha *et al*[23] found 95 per cent of so called 'skip metastases'

occurred in T3 and T4 tumours while Wood *et al*[24], Bilchik *et al*[25] and Kitagawa *et al*[26] all noted that their false negative cases occurred predominantly in T3/T4 patients. Furthermore, Ratanachaikanont *et al*[27] found significantly lower identification rates in these tumour categories and Yagci *et al*[28] documented lower sensitivities in those with advanced Dukes B cancers. The one exception to this very consistent trend has been the work of Paramo *et al*[29] who initially described a 0% false negative rate in their initial experience with predominantly T3 tumours. However, after further expanding their series (mostly by enlarging their numbers of T3 tumours), they found a 3% false negative rate[30] while, interestingly, neither report included any T4 cases.

Some other important studies have provided no meaningful details at all regarding T stage[31-39] and so do not lend themselves to be scrutinised in this fashion. However, given that they state no pre-selection criteria for their patients, it would seem likely that their patients were representative of typical presentation (which in the case of colon cancer is predominantly with transmural, node positive disease). Indeed, that the majority of studies include all comers in their validation studies is perhaps understandable given that most patients with potentially resectable tumours proceed to operation without particular consideration of tumour diameter or mural penetration. Nonetheless, inclusion of high proportions of more locally advanced tumours seems, both theoretically and empirically, to undermine the validation of the technique in this disease. While other factors such as operator experience and dye pharmacodynamics may, of course, also play a role, it would surely be of great interest to selectively, prospectively study sentinel node mapping in patients with early tumours. This would evidentially necessitate some means of preselection such as endoscopic ultrasound (proven efficacious in this tumour type as in other alimentary malignancies)[40] or by including for study only those with screen-detected cancers but would seem eminently feasible.

The second fundamental difference between SNM for cutaneous and breast tumours and that for colon cancer is in the overall intention of purpose. The fundamental principles ab initio diverge significantly, however, between the two as the intent of the technique when used in the former malignancies is to identify lymph node negative patients (in order to spare them from morbid lymph basin dissections) whereas in the latter it has focussed mainly on detecting lymph node positive patients (to identify those who have otherwise occult dissemination and who, therefore, could benefit from systemic therapy). Prognostic prediction (and, therefore, adjuvant therapy prescription) in colon cancer however is largely determined by lymph node involvement and recently much attention has focussed on the adequacy of nodal harvests (whether by surgeon resection or pathologist detection)[41]. It may well be, therefore, that the value of sentinel node identification and analysis is to confirm that lymphatic dissemination has not taken place in tumours likely to be of early stage in order to save the searching for sufficient numbers of nodes to prove this (in excess of 40 perhaps) that would otherwise be necessary[42]. From this viewpoint, the upstaging of some cancers that are conventionally node negative becomes an added bonus rather than the sole outcome to justify the effort involved. While such a hypothesis can obviously only be purely speculative at present, some proof of concept could perhaps be advanced by the early adopters of lymphatic mapping in colon cancer in examining the survival of their sentinel node negative patients to date in comparison to those deemed node negative by conventional means but with low nodal counts.

Finally, it is intriguing to question the widely held assumption that sentinel node mapping in colon cancer has no value in minimizing the operative morbidity associated with resection of colonic tumours[43]. This basic tenet has become so dogmatic that a sentence to this effect nearly always forms part of the introduction, discussion and/or accompanying editorials of publications concerning the topic[44]. The intent behind the standard operations performed for colonic cancer is, however, to achieve full lymph node basin clearance concomitantly with resection of the primary in every case ("en bloc" or radical resection). The fact that lymphatic drainage closely follows the arterial (rather then venous) regional blood supply is what prompts the level of proximal vascular ligation (a "high-tie") and it is this manoeuvre that then determines the extent of the segmental bowel resection required (in order to minimize the risk of ischemia of the residual bowel). In many cases, the magnitude of visceral resection provoked by radical lymphadenectomy is far in excess of what would be associated with curative surgery in terms of marginal clearance (colonic tumours rarely infiltrate more then 2 cm beyond the area of gross involvement and therefore a resection margin of 5-10 is considered appropriate)[45]. Although there is likely to be a therapeutic value in resecting nodes positive for metastatic disease in colon cancer, the main value of such clearance for truly lymph node negative patients can only be the gain of prognostic information for reassurance. If adoption of sentinel node mapping obviated the need for wide lymph basin clearance for intraperitoneal colon cancers, the potential benefits would include shortened operative times, reduced postoperative convalescence and, perhaps, improved bowel function[46] and diminished rates of anastomotic dehiscence[47]. Furthermore, the potential for ureteric, duodenal and (in the male) spermatic vessel injury would be greatly reduced if root mesenteric dissection became unnecessary as would the hazard of splenic laceration that occurs with mobilization of this flexure [often necessary after high ligation of the inferior mesenteric artery (IMA) to ensure tension-free anastomosis after radical left hemicolectomy for sigmoid tumours] and the risk of sexual impotence that may result if para-aortic nerve injury occurs during flush ligation of the IMA at its origin from the abdominal aorta. Lastly, if a localized resection of the bowel was all that was required, the facility by which excisional colonic cancer surgery is performed by minimally invasive means would be markedly enhanced (and the now arduous learning curve reduced). In short, the clinical significance of localized rather then

radical colectomy is not at present known, but cannot be assumed to be negligible.

The reasons why the basic principles regarding the validation and clinical utility of lymphatic mapping for colon cancer have become so fundamentally divergent from that of breast and melanoma is not entirely clear and, to date, have rarely been discussed. The simplest reason to suspect why it has occurred may be simply that the operative terminology has led to a prevailing mindset. As breast cancer surgeons always considered two operations for their patients (mastectomy/wide local excision and axillary node clearance) and melanoma surgeons always advocated wide excision and lymph basin dissection, perhaps the basic similarities would be easier appreciated if operations such as left hemicolectomy were to have more standardised names such as 'segmental colonic resection with mesenteric lymph node resection'.

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TOPIC HIGHLIGHT

Michael F Byrne, MD, *Series Editor*

A potpourri of pancreatic issues

In this issue of *World Journal of Gastroenterology*, we present a series of articles relating to various aspects of pancreatic disease. Overall, our understanding of the etiology of pancreatic disease is improving, and there is much more of a move towards reserving endoscopic retrograde cholangiopancreatography (ERCP) for cases in which endotherapy is likely and utilising other less invasive modalities such as EUS. In this series, Branch *et al* discuss the utility of ERCP in the evaluation and management of acute pancreatitis, focusing on the need to move away from diagnostic ERCP. With this in mind, Gerke *et al* present an update on the role of EUS in pancreatic disease. What we do for the truly idiopathic pancreatitis population has been the subject of a large body of literature over the last few years and is highlighted here by the paper of Enns *et al*. The perpetually contentious disease process of sphincter of Oddi dysfunction is explored here by Mitchell *et al* and provides some food for thought as to who should be doing manometry and which patients should be exposed to this potentially curative but also risky procedure. Byrne *et al* focus on the emerging burden presented by autoimmune pancreatitis, a condition undoubtedly missed in the past and often confused for pancreatic cancer. Freeman tickles our fancy with some revelations in to the pancreatic endocrine and exocrine manifestations of pancreatic disease. Finally, Meloche presents a delightful review of the current state of play in relation to pancreatic transplantation for type 1 diabetes mellitus.

This is a somewhat mixed bag of topics but hopefully serves to remind us that there are a number of considerations in pancreatic disease, that we have more therapeutic options, safer imaging, and that we have a lot more to learn.

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TOPIC HIGHLIGHT

Michael F Byrne, MD, *Series Editor*

Review of idiopathic pancreatitis

Jason Kihyuk Lee, Robert Enns

Jason Kihyuk Lee, University of British Columbia Department of Medicine, Vancouver, British Columbia, Canada
Robert Enns, University of British Columbia Department of Medicine, Division of Gastroenterology, St. Paul's Hospital, Vancouver, British Columbia, Canada
Correspondence to: Robert Enns, MD, FRCP, University of British Columbia Department of Medicine, Division of Gastroenterology, St. Paul's Hospital, Vancouver, British Columbia, Canada. renns@interchange.ubc.ca
Telephone: +1-604-6886332 Fax: +1-604-6892004
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Abstract

Recent advances in understanding of pancreatitis and advances in technology have uncovered the veils of idiopathic pancreatitis to a point where a thorough history and judicious use of diagnostic techniques elucidate the cause in over 80% of cases. This review examines the multitude of etiologies of what were once labeled idiopathic pancreatitis and provides the current evidence on each. This review begins with a background review of the current epidemiology of idiopathic pancreatitis prior to discussion of various etiologies. Etiologies of medications, infections, toxins, autoimmune disorders, vascular causes, and anatomic and functional causes are explored in detail. We conclude with management of true idiopathic pancreatitis and a summary of the various etiologic agents. Throughout this review, areas of controversies are highlighted.

Key words: Idiopathic pancreatitis; Recurrence; Etiology; Endoscopic retrograde cholangiopancreatography; Magnetic resonance cholangiopancreatography; Sphincter of Oddi dysfunction

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INTRODUCTION

Pancreatitis is a relatively common disorder with a myriad of etiologies all resulting in a common end result of inflammation within the pancreas. Acute pancreatitis (AP) results in acute inflammation typically presenting as abdominal pain with elevated levels of pancreatic enzymes[1]. Chronic pancreatitis (CP) is defined as a clinical syndrome of progressive inflammatory changes in the pancreas leading to permanent structural damage with subsequent impairment of both exocrine and endocrine function[2]. Chronic pancreatitis is often preceded by recurrent bouts of acute pancreatitis, however, occasionally it can present in a 'silent' fashion.

The label of "idiopathic pancreatitis" (IP) was originally designated to cases of pancreatitis wherein a diagnosis could not be made through a thorough history, physical examination, laboratory studies, and noninvasive imaging modalities such as abdominal ultrasonography/computerized tomography. Previously, this nomenclature had accounted for 8%-44% of cases being termed "idiopathic"[3-9]. Recent new laboratory and technological advances have been able to shred the enigmatic veils of IP to a point wherein with extensive evaluation it is possible to reveal the etiology in 79%-80% of patients previously labeled as having "idiopathic pancreatitis"[10,11]. More modern imaging investigations include, but are not limited to fine cut computerized tomography, endoscopic retrograde pancreatography, magnetic resonance pancreatography as well as endoscopic ultrasound.

EPIDEMIOLOGY

Longitudinal data on incidence trends report an increase in AP[12] which may be attributable to increases in all causes of pancreatitis as well as improved detection methods. The fatality rate for pancreatitis has continued to range between 3%-10%[13-18] despite a marked decrease in pancreatitis case fatality presumably secondary to early recognition of severity and complications as well as improved intensive care management[14,15]. Thomson[6] and others[7,17] have noted that in AP mortality rates are greater when the etiology is IP (14.1%)[6], compared with gallstone pancreatitis (7.2%)[6]. In addition, the clinical impact of IP is further highlighted by the fact that 40 percent (the largest subgroup) of all AP fatalities are attributable to IP[19, 20]. The incidence of IP ranges from 4.21 per 100 000[21] to as high as 45.33 per 100 000[9] depending on the population studied and the time in which data was collected with a trend toward high incidence in populations studied more recently[12,13,22]. The incidence appears to be

Table 1 Etiologies of acute or acute recurrent pancreatitis

Category	Agent/Diagnosis
Vascular	**Atheroembolism**
	Intraoperative hypotension
	Hemorrhagic shock
	Vasculitis (systemic lupus erythematosus and polyarteritis nodosa)
Infectious	**Viral**
	Mumps
	Coxsackievirus type B
	Hepatitis B
	Cytomegalovirus
	Herpes simplex
	Varicella-zoster
	HIV
	Rubella (probable)
	Bacterial
	Legionella
	Leptospira
	Salmonella
	Mycoplasma
	Brucella
	Mycoplasma
	Salmonella typhi
	Fungal
	Aspergillus
	Parasites
	Toxoplasma
	Cryptosporidium
	Ascaris lumbricoides
Trauma	Blunt or penetrating abdominal injury
	Post-ERCP pancreatitis
	ERCP sphincterotomy
	Manometry of sphincter of Oddi
	Iatrogenic operative complication
Metabolic	Hypertriglyceridemia (types Ⅰ, Ⅳ, Ⅴ)
	Hypercalcemia
	Hyperparathyroidism
Toxins	Ethyl alcohol
	Scorpion venom
	Methyl alcohol
	Organophosphorous insecticides
Medications	**Antimicrobial agents**
The following drugs were definitely associated with pancreatitis	Metronidazole, Stibogluconate, Sulfonamides, Tetracycline, Nitrofurantoin, Erythromycin, Isoniazid
	HIV Therapy
	Didanosine, Pentamidine
	Diuretics
	Furosemide, Thiazides
	Commonly used Gastroenterology Medications
	5-ASA, Sulphasalazine, Cimetidine, Ranitidine, Mercaptopurine, Proton pump inhibitors
	Cardiac Agents
	Procainamide
	Immunosuppressives or Chemotherapeutics
	L-asparaginase, Azathioprine, Cytosine arabinoside, Dexamethasone
	Neuropsychiatric Agents
	Valproic Acid, αMethyl Dopa
	Other Commonly Used
	Acetaminophen, Salicylates, Sulindac, Calcium, Ethinylestradiol, Norethindrone
Mechanical	Gallstones
	Microlithiasis and Biliary Sludge.
	Sphincter of Oddi dysfunction
	Pancreas divisum
	Annular pancreas
	Autoimmune pancreatitis
	Pancreatobiliary Tumours
	Cholodochocele
	Duodenal stricture or obstruction
	Ascariasis
Miscellaneous	Post ERCP
	Renal transplant
	Hyper IgG4 disease
Genetic	CFTR
	Serine protease inhibitor Kazal type 1 mutation
	Cationic trypsinogen gene PRSS1 mutation
Autoimmune	Sjogren's syndrome
	Primary biliary cirrhosis
	Renal tubular acidosis

equal between the two sexes and tends to increase with age in both before starting to plateau around 70 years[18].

Elucidating the etiology of pancreatitis, if possible is paramount as it guides therapy and may theoretically subsequently improve patient outcomes, thereby preventing relapses. Some studies have shown that over 50% of untreated patients with acute IP experience recurrent episodes[23-25]. This contrasts with other studies where only 1 of 31 patients with a first episode of unexplained AP suffered another attack during a median follow up of 36 mo[26]. These conflicting results may reflect different patient populations, perhaps even within the same category known as IP.

Aside from the initial objective to decrease acute patient mortality and morbidity, repeated insults to the pancreas may progress to chronic pancreatitis with irreversible morphologic and functional changes[2,24,27]. This concern typically results in aggressive investigation for those patients with more than one episode of AP. This approach is supported by a study by Kaw and Brodmerkel in a study that included 126 patients with two or more episodes of IP. They demonstrated that investigations including bile for microlithiasis, a secretin stimulation test, and sphincter of Oddi manometry (SOM) was able to clarify the etiologies in 79 percent of patients[11]. In this study they were able to offer 75% of these patients treatment that resulted in the absence of AP in over 60% of cases over a 30 mo follow-up period[11].

However there is ongoing debate against aggressive evaluation. The Kaw and Brodmerkel study confirmed previous morbidity data demonstrating that SOM is not a benign procedure and that complications occur[11]. Additionally, many aggressive techniques for diagnosing the etiology of pancreatitis, including SOM, may not be routinely available. Therefore the generalizability of this study has been brought into question.

This article will examine some of the etiologies that must be excluded prior to a diagnosis of IP. An attempt will be made to highlight the areas of controversy, and make suggestions based on the best available evidence. For the purpose of this IP article, gallstones and alcohol related pancreatic disease will not be discussed as their presentation is usually easily determined. For a more comprehensive list of all etiologies of pancreatitis please refer to Table 1.

PANCREATITIS ETIOLOGIES OFTEN LABELLED AS IDIOPATHIC PANCREATITIS

Microlithiasis and biliary sludge

Biliary sludge refers to the viscous suspension in gallbladder bile formed by modification of hepatic bile by the gallbladder mucosa that may contain small stones (< 5 mm in diameter)[28]. Microlithiasis refers to stones of < 3 mm in diameter and is often used interchangeably at times with "microcrystals" and sometimes with biliary sludge[28-30]. Microscopy of bile in patients with sludge often shows cholesterol monohydrate crystals, calcium carbonate microspheroliths, or calcium bilirubinate granules[31]. A known risk factor for the development of sludge typically includes prolonged fasting states as well as some antibiotics (ceftriaxone)[32]. Microlithiasis has been suggested to be the most common causes of IP[31,33] with a prevalence ranging from 6%-73%[11,25,31,33,34].

It has been demonstrated that treatment with cholecystectomy, endoscopic sphincterotomy, or ursodiol significantly reduces further attacks of IP[31,33]. Therefore it was inferred that in the absence of other identifiable risk factors, the presence of microlithiasis was enough to cause IP[11,26,35]. The caveat to interpreting these studies is that combined, they involved only 74 patients who had IP and treatments were clearly not double blinded[31,33].

An Indian study published earlier this year evaluating 51 patients with recurrent IP found that microlithiasis accounted for a mere 13 percent of these patients (using duodenal bile samples)[36]. In the setting of suspected biliary pancreatitis up to 88% of patients will have microlithiasis demonstrating the difference between these two patient groups[37]. It is speculated that there may be some gender, age, racial, and procedural differences in bile sample collection and analysis as these are not well standardized.

The pathogenesis of AP *via* microlithiasis remains unclear however it is thought that the microlithiasis may transiently impact the papilla, cause pancreatic duct obstruction and thereby pancreatitis[29,37,38]. The diagnostic workup for microlithiasis includes routine abdominal ultrasound (US) which has limited sensitivity when looking for stones less than 3 mm in diameter[28,39]. Endoscopic US (EUS) may be considered as well as it carries a lower risk of complications than endoscopic retrograde cholangiopancreatography (ERCP)[34,40-45]. On average, EUS is able to identify gallbladder sludge in up to 75 percent of IP cases[40-45]. All patients with recurrent IP should have microlithiasis excluded and there is even some evidence that it should be excluded for patients who have their first IP[41-43].

It remains to be clarified which method of bile collection for microscopic analysis is clinically the best and there are no standardized methods or recommendations at this time[46-48].

Cholecystectomy is recommended for patients with biliary sludge/microlithiasis once they recover from their episode of pancreatitis as it reduces the relapse rates[31,33]. In patients who are poor surgical candidates, ERCP with biliary sphincterotomy or ursodeoxycholic acid may be alternative forms of treatment[31,33].

Sphincter of Oddi dysfunction

At times referred to as hypertensive or fibrotic SOD, SOD causes diminished transphincteric flow of bile and/or pancreatic juice due to either organic obstruction (stenosis) or functional obstruction (dysmotility)[3,35,49]. SOD often causes recurrent pain with or without abnormalities of either hepatic/pancreatic profiles as well as duct dilation. It is thought that SOD causes pancreatitis as a result of bile reflux into the pancreatic duct or from pancreatic duct outflow obstruction[25,50]. SOD is considered to cause up to one third of all cases of IP[25,30,51,52].

In order to diagnose SOD, one often needs to perform sphincter of Oddi manometry (SOM)[53,54]. The diagnostic gold standard is a water-perfused catheter system that can be inserted into the common bile duct or pancreatic duct with the positive finding being a hypertensive sphincter of Oddi pressure greater than 40 mmHg[53]. Unfortunately whether ERCP in patients with suspected SOD is done for diagnostic SOM or therapeutic purposes it is undeniable that there have been high complication rates, particularly pancreatitis[51,55-59]. Additionally "severe" pancreatitis that is associated with death has even been quoted in 1-3% in SOM[15,25,51]. Despite this, ERCP with SOM has been advocated for the evaluation of recurrent IP[25,60]. In patients who have normal biliary manometry, pancreatic sphincter manometry may be reasonable to perform[61,62] which does carry a higher risk of pancreatitis[54] that may be reduced by aspirating through the middle port of the triple lumen manometry catheter[63].

Once the diagnosis of SOD is made, endoscopic sphincterotomy is the treatment of choice as it is believed to decrease the risk of recurrent pancreatitis[25,60,64-66].

The Geenen-Hogan (Milwaukee) criteria was devised to predict the overall probability of response to biliary sphincterotomy taking into account the presence of abnormal liver chemistries and dilated bile ducts[44]. On the basis of this, patients with pain and both abnormalities (type Ⅰ) are definitely recommended to have sphincterotomy as they have high response rates ranging from 90%-100% regardless of SOM[67,68]. Along this stratification, type Ⅱ patients have either abnormal liver chemistry or dilated bile ducts. If SOM is abnormal in type Ⅱ patients they will have a response rate to sphincterotomy of 60%-91%[64,67,69] and thus this would be a reasonable course of action. On the contrary, type Ⅲ patients do not have objective biliary abnormalities and even if they have abnormal SOM they have poor response rates of 6-58 percent[69-71], making sphincterotomy of questionable benefit (Table 2) .

If a diagnosis of pancreatic sphincter dysfunction is made in patients who have had biliary sphincterotomy the answer to who best benefits from pancreatic sphincterotomy is less clear. A study by Freeman *et al*[72] this year treated suspected SOD with biliary sphincterotomy with additional pancreatic sphincterotomy at initial or subsequent ERCP if there was abnormal pancreatic manometry in conjunction with pain refractory to biliary sphincterotomy, continuous pain, or a history of amylase elevation. In this study of 121 predominantly female (92%) and post cholecystectomy (87%) patients, all patients underwent biliary sphincterotomy while 40 percent also

Table 2 Sphincter of Oddi dysfunction (Geenen and Hogan Classification)

Biliary type	Pancreatic type
Type Ⅰ	**Type** Ⅰ
Biliary-type pain	Pancreatic-type pain
LFT elevation	Amylase/lipase elevation
CBD dilation	PD dilation
Delayed drainage	Delayed drainage
Type Ⅱ	**Type** Ⅱ
Biliary-type pain	Pancreatic-type pain
One or two of above criteria	One or two of above criteria
Type Ⅲ	**Type** Ⅲ
Biliary-type pain only	Pancreatic-type pain only

CBD: Common bile duct; LFT: Liver function tests; PD: Pancreatic duct.

underwent pancreatic sphincterotomy regardless of the modified Milwaukee biliary classification[72]. The result from this study was that a positive response at final follow up was reported in 69 percent of patients and that this response was not significantly different between biliary types Ⅰ, Ⅱ, and Ⅲ[72]. Freeman *et al*[72] concluded that patient characteristics of pancreatic manometry, delayed gastric emptying, daily opioid use, and age < 40 were significant as predictors of outcomes as opposed to the Milwaukee classification. Indeed it is becoming more accepted that a lack of improvement after biliary sphincterotomy may be representative of a failure to relieve pancreatic sphincter pressure[61,62,73-77].

Noninvasive strategies such as a low-fat diet, analgesics, anticholinergics, calcium-channel blockers, nitrates, and proton pump inhibitors are unfortunately seldom effective[78,79].

Pancreas divisum

In Pancreas divisum it is thought that 80%-95% of pancreatic juice volume flows *via* the dorsal duct through the smaller minor papillary orifice *via* the dorsal duct of Santorini as opposed to the more common route through the major papilla *via* the ventral duct of Wirsung[66]. It is postulated that the mechanism is that of relative minor papilla outflow obstruction leading to pancreatitis[23,80-82].

Pancreas divisum occurs as an anatomic variant in 5%-7.5% of patients[23,82]. There are those who are skeptical of the obstructive theory as fewer than 5% of patients with pancreas divisum develop pancreatitis[83-86]. It also appears that the incidence of pancreas divisum is the same in patients with and without pancreatitis[86]. Nonetheless there is consensus that while most individuals with pancreas divisum live normally, a few unfortunate patients are predisposed to develop recurrent AP[23-25,35] and that it accounts for 20 percent of the IP cases[23-25].

One explanation of why some patients with pancreas divisum are more likely to be affected was the smaller than usual minor papillary orifice causing a disproportionately high intrapancreatic dorsal ductal pressure especially during times of active secretion. In this situation a cascade of inadequate drainage leading to ductal distension and eventually pancreatitis could occur[23,87-89]. This theory is supported by at least one surgical study that demonstrated relief of pain as well as diminished attacks of AP with sphincteroplasty[87].

Recently, Choudari *et al*[90] found that prevalence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations was similar in patients with pancreas divisum with recurrent acute pancreatitis and true idiopathic recurrent acute pancreatitis. Interesting, this study also found that the prevalence of CFTR gene mutations in patients with pancreas divisum but without recurrent AP was similar to controls without pancreatitis[90]. The significance of CFTR will be discussed in more detail under "Genetic Causes and Contributions" below, however, these genetic studies call into question the true role of pancreas divisum in IP.

At present, it is accepted that patients who present with severe pancreatobiliary type symptoms or recurrent IP should however be evaluated for pancreas divisum[35,48]. The diagnosis of divisum can be made either by ERCP or magnetic resonance cholangiopancreatography (MRCP)[91,92]. ERCP may be preferred to conventional MRCP as it has a higher sensitivity and specificity[91,92] and can offer therapy albeit with a complication rate that is higher than in MRCP. Secretin may be given intravenously to aid in finding the minor papillary orifice at ERCP[93], finding functional outflow obstructions[94-96], and assessing the relative obstruction of the minor papilla[94-96]. In settings where secretin is either contraindicated or does not produce a visible increase in pancreatic juice flow, a dilute (1:10) methylene blue solution can assist in the location of the minor papilla[97]. Confirmation is recommended to rule out pseudodivisum caused by a tumor.

An emerging area is the role of EUS as it is a minimally invasive tool for the general workup of IP and recurrent IP[48]. It appears that EUS is able to detect pancreas divisum but more research in comparing ERCP to EUS specific to divisum is needed before recommendations are made.

In the setting of recurrent AP, once divisum is diagnosed, ERCP therapy to relieve minor papillary obstruction is recommended to decrease the rate of recurrent pancreatitis[98-101], particularly in patients with a dilated pancreatic duct who may be the most likely to benefit from therapy[98]. The rate of decrease of recurrent pancreatitis is quoted to range from 70%-90%[99,100] with a 40%-60% response rate in patients with CP also demonstrated[102]. The ERCP therapy involves a minor papillotomy usually with temporary stenting[98-101,103]. In one study, more favorable long-term results were achieved with minor papilla sphincterotomy than with repeated stenting in terms of both recurrence and fewer complications (25% *vs* 44% respectively)[98]. Some endoscopists are particularly cautious about endoscopic stenting as the procedure may cause permanent damage to the pancreatic ducts, possibly promoting the development of chronic pancreatic disease[104,105].

Anomalous union of pancreaticobiliary duct

Anomalous union of pancreaticobiliary duct (AUPBD) is defined as an anomalous junction of bile duct and

pancreatic duct at an abnormal proximal site of these ducts[35]. The precise location of the junction is outside the duodenal musculature and independent of the sphincter of Oddi. This independence from the contractility of the sphincter of Oddi results in regurgitation of the pancreatic juice into the biliary tract and vice versa[106]. AUPBD is frequently associated with choledochal cysts which in itself has been linked to IP (see below)[107] and is more common in East Asia[35].

It is thought that in AUPBD reflux of bile into the pancreatic duct may cause recurrent AP[106,108] and that in cases with a dilated common channel the stasis of pancreatic secretion can occur in the common channel leading to pancreatitis[109,110]. Moreover it has also been found that SOD (see above) is sometimes associated with AUPBD[111].

The diagnosis of AUPBD is usually made during ERCP evaluation for IP when one notes a pancreatic duct and choledochus connecting with a long common channel of > 15 mm in adults[112]. Similarly AUPBD is also seen during MRCP[91]. The length of the common channel alone is not an absolute diagnostic criterion as other factors like the form of union, direction, and age and stature of patients need to be considered[108,113]. An alternate method of diagnosis, more importantly a 'clue' to the presence of AUPBD, may be the presence of high amylase levels in aspirated bile[114,115].

In AUPBD, endoscopic biliary sphincterotomy has been shown to prevent further attacks of AP[112] by a postulated mechanism of decreasing resistance at the major duodenal papilla[35]. Due to the fact that AUPBD has a propensity to increase gallbladder and biliary duct cancer[114,116-118], prophylactic cholecystectomy has been recommended in these patients[116]. As well, at this time there is some evidence that patients with AUPBD and choledochal cyst should have an extensive resection of the extrahepatic bile duct inclusive of the cyst in hopes of preventing development of biliary duct cancer[108] which may be relevant in 5% of patients[116]. It is reiterated that the understanding of AUPBD, its pathophysiology and therapy is very limited[35].

Choledochocele

Choledochocele is classified as a type Ⅲ choledochal cyst that represents a prolapse or herniation of the intramural segment of the distal common bile duct into the duodenal lumen which may be either congenital or acquired[35,119]. Pancreatitis and recurrent AP is an important complication occurring in choledochoceles occurring in 12%-30% of patients[120,121]. Choledochoceles do not represent a common cause of IP, especially in adults[25,35,120].

It is thought that the choledochocele may create an obstruction to the pancreatic duct intermittently when the choledochocele becomes distended and that this may lead to reflux of bile into the pancreatic duct[121-123].

A characteristic "bulging" appearance of the papilla at ERCP and a soft "pillow" sign with pressure applied at the catheter tip suggests a choledochocele[120,124]. A CT or US in isolation may miss this diagnosis[120]. The role of MRCP in adults to diagnose this is still undefined[91,125,126].

Treatment can be initiated with endoscopic sphincterotomy combined with "unroofing" of the choledochocele with a papillotome with the goal of creating effective drainage of bile and pancreatic juice[120,127]. Patients that fail this treatment may be considered for a surgical sphincteroplasty[128]. Reports in the surgical literature about choledochal cysts in general recommend a single stage surgery usually comprising of complete cyst resection, cholecystectomy and Rou-en-Y hepatojejunostomy[129-131]. Malignancy is noted in 3-5 percent which may in some cases warrant prophylactic surgery[131,132].

Annular pancreas

Annular pancreas is a congenital condition that represents a band of pancreatic tissue partially or completely encircling the duodenum that is usually at the level of or immediately proximal to the major duodenal papilla[133]. It is thought that this defect occurs in utero due to the failure of the ventral bud to rotate with the duodenum[133]. This abnormality is rare and is detected in 1/7000-1/20 000 autopsies[133,134] and in 1/1500 ERCPs[135-137].

Clinically it usually manifests in childhood with intractable vomiting[136,137] and is thought to result from descending duodenal narrowing leading to duodenal obstruction or recurrent AP[138]. In adults annular pancreas may present with abdominal pain, acute recurrent IP, CP, peptic ulcer disease, postprandial fullness, vomiting, or biliary obstruction[137,139]. Associated congenital anomalies such as Down's syndrome, cardiac defects, tracheoesophageal fistula, Meckel's diverticulum, and imperforate anus may be present[133,136]. If the diagnosis is made in adults it usually occurs between the ages of 20 to 50[140].

From a diagnostic view, barium studies, abdominal CT, or MRCP may suggest the diagnosis but an ERCP is recommended for confirmation[135,136,141]. A typical ERCP is that of the duct of the pancreatic annulus encircling the duodenum[24] and in one third of cases a pancreas divisum is also present[139]. The annular duct may communicate with the central duct but rarely drains into the dorsal duct, common bile duct, or independently into the duodenum[135,139]. In cases where ERCP may not be feasible, an EUS may offer an alternative means of diagnosis[142]. Recently some data has also become available on the role of MRCP which appears encouraging as a non-invasive method of diagnosis[143-145].

Surgery is the procedure of choice in patients in whom symptoms can be attributed to annular pancreas with the goal to relieve duodenal or gastric outlet obstruction[137,146].

Pancreatobiliary tumors

Any mass that obstructs the main pancreatic or biliary ducts, benign or malignant can result in acute pancreatitis. It has been estimated that 5%-14% of patients with pancreatobiliary tumors, benign or malignant, present with apparent IP[147-150]. Pancreatic cancer should be suspected in any patient older than 40 years with IP especially with a prolonged or recurrent course[3,5]. Neoplasia should be suspected in a patient with weight loss, steatorrhea, ductal dilation, new onset diabetes, or evidence of a solid

or cystic pancreatic mass[24,149,150]. In younger patients lesions such as curable islet cell tumors should be in the differential whereas in the elderly they may have potentially curable lesions such as cystic neoplasms[24].

It is well known that CT, MRI, ERCP, and EUS have a role in identifying pancreatobiliary neoplasms[151-154]. It is recommended that if there is any clinical suspicion of malignancy that aggressive investigation including ERCP be performed even on the first attack of IP in older patients. In patients who are less than 40 years old, a CT may be sufficient in first attacks of AP[24]. If a malignancy is suspected, EUS is a favorable technique that can be used for diagnostic and staging purposes[155,156] especially when combined with fine needle biopsy[157,158].

Intraductal papillary mucinous neoplasm of the pancreas (IPMN) is a distinct pathologic entity formed of papillary proliferations of mucin-producing epithelial cells with or without excessive mucus production and or cystic dilation of the pancreatic duct. IPMN represents a precancerous lesion with a well described adenoma and carcinoma sequence that causes recurrent acute IP with symptoms suggestive of chronic obstructive pancreatitis due to intermittent obstruction of the pancreatic duct with mucus plugs[159]. It is worth noting this entity has an insidious nature and lack of awareness often delays diagnosis[160-163]. On ERCP, in cases of main duct involvement, the papilla is patulous and resembles a "fish-eye" frequently with mucus extruding from the orifice[161,164,165]. In terms of management, since the ten-year actuarial risk of high grade dysplasia and invasive cancer is significant[166] surgery is usually recommended, particularly for main duct disease[159,167-169]. It should also be noted that patients with IPMN may be at increased risk for extrapancreatic malignancies and because of this gastricadenocarcinoma and colorectal cancer should be screened for using endoscopy[170,171]. Patients with branch type disease may be at a lower risk of malignancy and theoretically can be monitored in regards to mural wall thickening, size of branch cystic lesion and tumor markers.

Cystic pancreatic tumors including IPMN, serous cystadenomas, mucinous cystadenomas, and mucinous cystadenocarcinomas, can be premalignant or malignant and surgery is generally indicated[172-174]. Tumor markers such as CA 19-9, CA 15-3, CA 72-4, and carcinoembryonic antigen in aspirated cystic fluid along with fluid viscosity and amylase level may be used to increase the diagnostic yield of cyst fluid cytology[175-179].

Ampullary adenomas are premalignant and in general, indicate the need for close monitoring and eventual removal[180,181]. In general, ampullary tumors have a more favorable prognosis than pancreatic tumors and pancreatoduodenectomy has historically been recommended[182-184]. An emerging development for ampullary tumors is the use of endoscopic techniques such as endoscopic ampullectomy for management of patients with small benign lesions or for carcinoma *in situ*[185-188] which appears to be safe and efficacious on long term follow up[188]. If endoscopic management is selected it has been recommended that surveillance and random biopsies be performed[24].

GENETIC ASSOCIATIONS AND CAUSES

An exciting development is the recognition of genetic mutations that are associated with pancreatitis.

CFTR

The cystic fibrosis transmembrane conductance regulator (CFTR) and its gene mutations cause cystic fibrosis in an autosomal recessive pattern. It is well established that CFTR mutations cause disease of the exocrine pancreas[189]. The CFTR gene encodes a chloride-channel protein that is regulated by cAMP. Bicarbonate ion is secreted into the duct lumen by the action of CFTR in the apical membranes and this sets up a gradient in which water follows[190]. In the absence of CFTR, the pancreatic duct cells cannot secrete fluid and bicarbonate and hence CF of the pancreas develops[191].

The actual mechanism that mutations in the CFTR gene causes recurrent AP or CP[90,192-194] is unknown. It may be that mutant CFTR channels are inefficient at flushing digestive enzymes out of the pancreatic duct and thereby limiting the major mechanism that prevents trypsin-associated injury within the pancreatic duct[195]. Along the same lines, it may be that mutant CFTR can limit bicarbonate secretion that can interfere with trypsin activation[195]. What is clear is that CFTR mutations have a demonstrated increased incidence in patients with CP, IP, and alcohol induced CP[194,196-201]. One recent study of 381 patients diagnosed with either CP or recurrent IP that used expansive CFTR genotyping showed mutant CFTR genes in 11% (43/381) of these patients[195]. This frequency is in keeping with previous reports of 28%-45% of IP patients being either heterozygous or homozygous for a CFTR gene mutation[202,203]. One study in Poland showed that the frequency of mutations in CFTR alleles was similar to controls (4.9% *vs* 5%, $P = 0.587$) but this study only looked at 3 CFTR defects[204].

Testing for this gene mutation may be considered in younger patients presenting with recurrent IP who have a positive family history of cystic fibrosis or IP[66,195,205]. If testing is to be done, a broad mutation panel to look for a multitude of mutations of CFTR is recommended[195].

SPINK1

The N34S mutation of the serine protease inhibitor Kazal type 1 (SPINK1) has been reported to be strongly associated with IP and hereditary/familial pancreatitis[206-208]. SPINK1 is a specific trypsin and trypsin activated trypsin like inhibitor expressed within the pancreas that provides a defense against prematurely activated trypsinogen[209,210]. It has been suggested that SPINK1 mutations are disease modifiers in that they lower the threshold for pancreatitis from other genetic or environmental factors[207]. For example this mutation was found in one study to be associated with CP and recurrent IP in 7.7% and 10% respectively[204]. Likewise, a larger study showed that 15.7% of patients with CP or recurrent IP carried at least one SPINK1 mutation[195].

A genetic test for SPINK1 should be done in any younger patient presenting with recurrent AP or CP and a family history of IP or CP-particularly if they have had

prior negative workup with ERCP. When genetic testing is done, it is advised to perform a concomitant CFTR and PRSS1 mutation screen[195].

Cationic trypsinogen gene PRSS1

Two missense mutations R12211[211] and N291[212] in the human cationic trypsinogen gene [protease serine 1 (trypsin 1); PRSS1] were first detected in patients with hereditary pancreatitis and appear to manifest as autosomal dominant mutations[213]. Since the discovery of the first two genes many other PRSS1 mutations have been reported[214-219]. Pathophysiologically this mutation leads to impaired trypsin autolysis and degradation, impairment of SPINK1 defense mechanism, and promotion of auto-activation of trypsinogen all of which can cause AP and lead to CP[211,220].

Symptoms typically arise in childhood but may be delayed until the mid 30 s[221]. These usually include symptoms associated with CP[221]. The lifetime risk of pancreatic cancer is 40% and reportedly 75% with paternal inheritance[222]. Whereas in cases of CFTR and SPINK1 are associated with IP and CP, PRSS1 defects seem to be causative for AP[204].

As in CFTR and SPINK1, the diagnosis is usually suspected if younger patients with affected family members present with CP or recurrent IP. Complications of CP are usually dealt with endoscopically but given the high risk of malignancy surgical resection may be preferred[24].

Celiac disease

The frequent familial occurrence and the remarkably close association with the HLA-DQ2 and/or DQ8 gene locus suggests that celiac disease as an immune disorder that is triggered by an environmental agent, gliadin, in genetically predisposed individuals[223,224]. Intriguingly, recurrent pancreatitis can be caused by celiac disease[225,226]. The mechanism appears to be duodenal inflammation and associated papillary stenosis causing pancreatitis[225]. Endoscopic treatment is recommended at this time to relieve the obstruction.

There are consensus guidelines to the role of genetic testing in IP although they are evolving as our knowledge expands in the area[227].

MEDICATIONS

The list of medications that are associated with or that cause pancreatitis is increasing[228-230]. In one German study 1.4% of all acute pancreatitis was related to medications[231]. Another study reported an even lower association of 0.3%[232]. Drugs with the highest incidence of pancreatitis are azathioprine and mercaptopurine (incidence, 3 to 5%)[233] and didanosine (23%)[234].

Most medication related to pancreatitis is due to idiosyncratic response or a direct toxic effect. A high index of suspicion and astute drug history is crucial for making the diagnosis.

A few areas of medications causing pancreatitis will be highlighted. Firstly, physicians should be aware of a recent development showing an association of proton pump inhibitors and pancreatitis[235-237]. Secondly, some medications, such as pentamidine, valproic acid, and didanosine, appear to cause injury weeks to months after exposure, possibly through the accumulation of a toxic metabolite[238]. Hypersensitivity reactions have been implicated in other drugs, such as azathioprine, mercaptopurine, metronidazole, aminosalicylates, and sulfonamides, and these drugs characteristically lead to pancreatitis within one month after exposure[238]. Lastly, some medications like acetaminophen may cause pancreatitis with a single dose[239].

A complete list of medications that are classified as being definitely associated with pancreatitis derived from multiple references is shown in Table 1[228-230,240-242].

TOXINS

Toxins that have been linked to pancreatitis include the most common alcohol. The more deadly methanol can also cause AP[243]. Although exceedingly rare scorpion bites from Trinidad have been known to cause AP[244,245]. Another rare cause of IP is organophosphorous poisoning[246].

INFECTIOUS CAUSES

A plethora of infectious agents have been associated with AP[247]. A full list of definite and probable etiologic agents is provided in Table 1 from data gathered from Parenti *et al*[247]. It is unclear how often an infectious etiology is responsible for IP but many appear to be associated with it[247].

A clinician must be suspicious of an infectious etiology in the characteristic syndrome caused by the particular infectious agent notwithstanding that this was evident in only 70% of patients in the prior review for definite cases[247]. A routine search for an infectious cause in IP is not recommended unless there is a strong clinical suspicion.

Ascariasis is an important cause of infectious obstructive AP in India where it is the second most common cause of AP[248]. At times this may also present with associated biliary tree infestation requiring endoscopic decompression[249].

An infectious group worth discussing in greater detail is HIV infections and their relation to AP[250]. One large series revealed that 4.7% of hospitalized patients with HIV had AP[251]. While primary HIV infection itself can be a cause of AP[252,253] it is more commonly attributable to a complication of medications taken as part of HIV treatment or medications for opportunistic infections such as Pneumocystis carinii and Mycobacterium avium-intracellulare[250].

METABOLIC

Hypertriglyceridemia

Serum triglyceride concentrations above 11.3 mmol/L are capable of causing AP although admittedly the pathogenesis of inflammation is unclear[254]. It is thought that hypertriglyceridemia represents 1.3%-3.8% of AP

cases[255]. The incidence has been best defined in children with inherited disorders of lipoprotein metabolism that is associated with severe hypertriglyceridemia which are 35%, 15%, and up to 40% in hyperlipidemia types Ⅰ, Ⅱ, and Ⅴ respectively[256,257]. Other acquired causes of hypertriglyceridemia include obesity, diabetes mellitus, hypothyroidism, pregnancy, estrogen or tamoxifen therapy, glucocorticoid excess, nephritic syndrome, and beta blockers[258-260,255].

Drug-induced disease is more likely to occur in patients with underlying hypertriglyceridemia[256]. It is important to not neglect the lactescent serum as it is a vital clue to the diagnosis[255].

Controversy surrounds the contribution of hyperlipidemia in causing AP in alcoholics[261] but it is thought that in most alcohol abusers the moderate elevations of triglyceride levels are transient and likely to be an epiphenomenon rather than a causative agent of pancreatitis[262].

Hypercalcemia

Occurring in uncommon frequency, hypercalcemia of any cause is a known cause of AP[263,264]. Postulated mechanisms include calcium deposition in the pancreatic duct and calcium activation of trypsinogen within the pancreatic parenchyma[265,266].

There are questions however on hyperparathyroidism, a cause of hypercalcemia, and the link to pancreatitis rose in one large study of 1153 patients that found that AP occurred in only 1.5 percent of patients that was of a statistically non significant difference from that of the general population[267]. This finding was mirrored in two other studies[268,269]. This is still an open controversy however as other studies have supported at least an association and a significantly increased relative risk of AP in patients with hyperparathyroidism[270,271]. Until the debate is resolved it is recommended that the parathyroid be checked in recurrent IP if hypercalcemia is present.

VASCULAR DISEASE

Pancreatic ischemia is an uncommon but an established cause of pancreatitis reported in: (1) vasculitis (systemic lupus erythematosus, polyarteritis nodosa, and microscopic polyangiitis)[272-274], (2) atheroembolism[275,276], (3) hypotension and shock[277-279].

While it is true that most patients have mild attacks of pancreatitis secondary to ischemia, fatal necrotizing pancreatitis is a rare occurrence[277].

TRAUMATIC CAUSES

Blunt or penetrating abdominal injuries can cause pancreatitis although it is incredibly rare given the retroperitoneal location of the gland[280]. Types of trauma that cause pancreatitis can range from a mild contusion, severe crush injury, or transection of the gland[280].

Post ERCP pancreatitis remains the commonest severe complication of ERCP found to occur in 5%-7% of patients in a recent review[281]. It may be possible that prophylactic stenting of the pancreatic duct in selective cases and minimally traumatic cannulation techniques may prevent some cases[281] and indeed this has been shown to be cost effective in high risk patients[282]. Octreotide infusion does not prevent ERCP-induced pancreatitis or affect serum amylase levels[283]. Likewise, transdermal glyceryl trinitrate did not improve the rate of success in ERCP cannulation or prevent post-ERCP pancreatitis in either average or high-risk patient groups[284]. Allopurinol at high doses has been shown in a prospective randomized trial to lower the risk of post-ERCP pancreatitis[285] but this study requires further verification as other studies have shown no benefit[285,286]. Risk factors for post-ERCP pancreatitis have been well described[287].

AUTOIMMUNE CAUSES

Autoimmune pancreatitis is a more recently described type of chronic pancreatitis characterized by an autoimmune inflammatory process in which prominent lymphoplasmacytic infiltration with associated fibrosis of the pancreas causes organ dysfunction[288-290]. The reported prevalence of autoimmune pancreatitis is between 5 and 6% of all patients with chronic pancreatitis[291]. A series from the United States shows that 11% of patients (27 of 254) with chronic pancreatitis received a diagnosis of autoimmune pancreatitis based on histological findings[292].

Immunologic abnormalities including hypergammaglobulinemia, elevated serum IgG4 levels, and the presence of autoantibodies against carbonic anhydrase and lactoferrin are important markers of the disease[288]. In particular, autoantibodies against lactoferrin and carbonic anhydrase Ⅱ have been identified as potential serologic markers of autoimmune pancreatitis[293,294]. The finding of increased serum IgG levels or the presence of autoantibodies is supportive of the diagnosis, whereas an elevated serum IgG4 level is nearly diagnostic[288].

CP has also been found to be in association with Sjogren's syndrome, primary biliary cirrhosis, and renal tubular acidosis[295,296]. The diagnosis is usually based on clinical suspicion and serum autoantibody to a pancreatic antigen previously discussed. Inflammatory bowel disease is also occasionally associated with autoimmune pancreatitis[297].

Differentiating the focal form of autoimmune pancreatitis rather than pancreatic carcinoma can be very difficult on the basis of CT imaging only[288]. Diffuse pancreatic-ductal narrowing is highly diagnostic of autoimmune pancreatitis[288,289,291]. There are also nonspecific endoscopic findings in the stomach or colon in patients with autoimmune pancreatitis, foci of slightly pale, thickened mucosa with loss of visible vascular pattern were observed in some cases[298]. The role for MRCP is at this time undefined[288].

EUS is a key tool in the diagnosis of autoimmune pancreatitis and its differentiation from other pancreatic diseases. The most common finding on endoscopic ultrasonography is diffuse or focal pancreatic enlargement along with a diffusely hypoechoic parenchyma, similar to findings on transabdominal ultrasonography[299,300].

The use of corticosteroid therapy is not mandatory in autoimmune pancreatitis, as there are reports of the spontaneous resolution of a pancreatic mass, stricture, and jaundice[301,302]. When steroids are given the response is often dramatic and can be monitored *via* clinical, laboratory and radiological parameters[303-306].

MISCELLANEOUS CAUSES

Recent reports of a new disease known as hyper IgG4 disease has been linked to IP[307] but more data is required before further comment. It is unclear if this is an entirely separate entity from autoimmune pancreatitis.

In renal transplant cases pancreatitis can occur as a result of the procedure itself[308], immunosuppressant medications (see above 'Medications'), opportunistic infections[309] or through allograft pancreatitis[310].

CHRONIC PANCREATITIS

The aforementioned etiologies of IP can all theoretically lead to CP. The diagnosis is best made after considering the results of ERCP, pancreatic function tests, and EUS[24].

It has been suggested that pancreatic function testing may help establish the diagnosis of CP at an earlier stage[311]. It appears based on recent evidence that EUS may be the most sensitive test[311,312] for CP diagnosis with the caveat of false positives[313].

Mortality rates for CP are 3-4 times greater than in controls[314,315]. Pancreatitis in CP accounts for 20 percent of mortality but mortality is usually from non-pancreatic causes[314,315]. CP carries with it many complications such as pancreatic duct strictures, stones, pseudocyst, fistulas, pseudoaneurysm, or ascites[316-320].

TRUE IDIOPATHIC PANCREATITIS

In spite of extensive systematic investigations and exhaustive efforts, there will be patients with true IP (TIP). Recommendations are difficult to make given the heterogeneity of studies that have evaluated this problem. Generally there are 4 therapies that might be applicable in TIP taking into account that TIP most likely represents a group of heterogeneous disorders: antioxidants, ursodeoxycholic acid, pancreatic enzymes, and somatostatin or its analogue octreotide.

Antioxidants (e.g., vitamin C, and E, beta carotene) have been shown to reduce the pain involved in IP[321]. It is postulated that patients with AP or CP may have a deficiency in antioxidants either locally within the pancreatic parenchyma or systemically[322]. A cocktail of antioxidants including 600 μg of selenium, 9000 IU of β-carotene, 0.54 g of vitamin C, 270 IU of vitamin E, and 2 g of methionine daily[321] was shown to have a statistically significant benefit in reducing attacks of pancreatitis. It is again stressed that these trials have limited power since the above study evaluated only 28 patients[321].

In patients who continue to have attacks of pancreatitis despite having cholecystectomy or endoscopic sphincterotomy, or patients with contraindications to surgical and endoscopic treatment, maintenance therapy with ursodeoxycholic acid has also been suggested with some benefit[31,323].

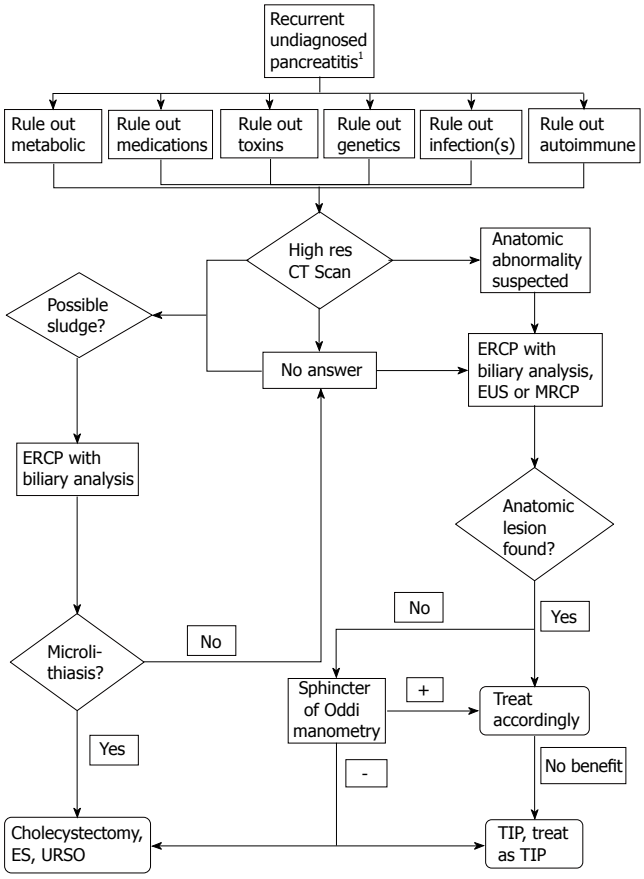

Figure 1 [1]To arrive at the label of recurrent undiagnosed pancreatitis, a history, physical exam, routine laboratory investigations, chest radiograph, abdominal ultrasound, and or computed tomography must fail to find the etiology of pancreatitis. Although sludge may be picked up on transabdominal ultrasound, the low sensitivity precludes exclusion of diagnosis of microlithiasis. ES: Endoscopic sphincterotomy; TIP: True idiopathic pancreatitis; EUS: Endoscopic ultrasound; CT: Computed tomography; URSO: Ursodeoxycholic acid.

Pancreatic enzyme therapy has recently been reviewed and the efficacy in IP or recurrent AP or CP is small[324]. However, given the low risk of enzyme therapy it has been suggested as a therapeutic trial in treating CP and IP[325,326].

Somatostatin or its analogue octreotide has been postulated to reduce pancreatic secretion and thereby be of benefit in the treatment of relapsing pancreatitis. This therapy entails either a continuous infusion or frequent injections in the past[327]. A 1 mo depot injection has recently become available and may make this therapy more attractive but data is limited with existing data on octreotide and somatostatin focused on altering outcomes during an index hospitalization of severe pancreatitis rather than preventing subsequent attacks[327].

APPROACH TO RECURRENT IP

The authors of this review propose the following systematic approach for patients who have had more than one attack of IP. The following is not validated and is meant to be only used as a guide. For specific details please refer to the aforementioned sections (Figure 1).

CONCLUSION

True IP is declining as knowledge and technology advances. IP has been found now to represent a myriad of etiologies that have been elucidated with the advancement of laboratory and endoscopic studies. It is thought that a thorough workup of these cases should reveal an etiology in up to 80% of cases. If genetic screening is applied it is suspected the etiologies may be explained in a much higher percentage of cases. There are still many controversies surrounding some of the IP etiologies and full consensus agreement is lacking in many areas. This review comprehensively outlined the latest evidence in the etiology, pathogenesis, diagnosis, and treatment strategies. It is felt that establishing a diagnosis is key, for it has the potential to direct management. It is recognized, however, that for many of the disorders (particularly genetic abnormalities) there are limited therapies. Elucidating all the causes of IP is a challenge that needs to be faced so that patients can avoid unnecessary morbidity and mortality from subsequent invasive testing.

The treating physician should perform a detailed history to rule out medication, metabolic, and toxin related etiologies. Moreover a detailed history should raise the possibility of genetic associations and causes as well as infectious etiologies so that further testing can be best directed. Celiac disease, vascular disease, and autoimmune causes of pancreatitis should be considered in evaluating the optimal approach to IP.

The most common causes of IP include microlithiasis and biliary sludge, sphincter of Oddi Dysfunction, and anatomic abnormalities. When there is any risk or suggestion of malignancy either as a cause or as an association of the etiology, as in choledochoceles, appropriate management including the necessary diagnostic workup and consideration of surgical excision when the diagnosis is made needs to be considered. In the aforementioned causes it is clear that both imaging techniques such as MRCP, EUS, high resolution CT, and interventional techniques as ERCP play their respective roles.

Indeed with further research additional causes of IP and associations of IP will be revealed. Even with exhaustive efforts and considering all the etiologies in this article there will still be a few patients who will continue to have true IP. In these patients non specific therapy including pain control is often unfortunately the only option. Certainly further advances in pancreatitis will be welcome.

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TOPIC HIGHLIGHT

Michael F Byrne, MD, *Series Editor*

Role of endoscopic retrograde cholangiopancreatography in acute pancreatitis

Karen R Canlas, Malcolm S Branch

Karen R Canlas, Malcolm S Branch, Division of Gastroenterology and Hepatology, Duke University Medical Center, Durham, North Carolina 27710, United States
Correspondence to: Malcolm S Branch, MD, Division of Gastroenterology and Hepatology, Duke University Medical Center, DUMC Box 3662, Durham, NC 27710, United States. branch003@mc.duke.edu
Telephone: +1-919-6843787 Fax: +1-919-6818785
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Abstract

Endoscopic retrograde cholangiopancreatography (ERCP) is a useful tool in the evaluation and management of acute pancreatitis. This review will focus on the role of ERCP in specific causes of acute pancreatitis, including microlithiasis and gallstone disease, pancreas divisum, Sphincter of Oddi dysfunction, tumors of the pancreaticobiliary tract, pancreatic pseudocysts, and pancreatic duct injury. Indications for endoscopic techniques such as biliary and pancreatic sphincterotomy, stenting, stricture dilation, treatment of duct leaks, drainage of fluid collections and stone extraction will also be discussed in this review. With the advent of less invasive and safer diagnostic modalities including endoscopic ultrasound (EUS) and magnetic retrograde cholangiopancreatography (MRCP), ERCP is appropriately becoming a therapeutic rather than diagnostic tool in the management of acute pancreatitis and its complications.

Key words: Endoscopic retrograde cholangiopancreatography; Acute pancreatitis

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INTRODUCTION

Most pancreatologists accept the 1992 Atlanta Symposium definition of acute pancreatitis as an acute inflammatory process of the pancreas with variable involvement of other regional tissues or remote organ systems[1]. It is recognized that the underlying process is reversible and the gland returns to normal once the injury resolves. The annual incidence is estimated to be 17 per 100 000 in the United States[2]. Over 333 000 hospital admissions and 911 000 physician visits in the United States each year are due to acute pancreatitis[2]. The most common causes in US adults are gallstone disease and excessive alcohol use, although clinically detectable pancreatitis never develops in most persons with these risk factors[3].

The pathogenesis of acute pancreatitis involves the inappropriate activation of trypsinogen to trypsin in excessive quantities to overwhelm the mechanisms of elimination within the pancreas. Activation of these digestive enzymes causes pancreatic injury and an intense inflammatory response which results in microcirculatory injury, leukocyte chemoattraction, release of cytokines and oxidative stress. The release of pancreatic enzymes damages the vascular endothelium, the interstitium and acinar cells. Microcirculatory changes and progressive ischemia occur, which increase vascular permeability and leads to edema of the gland, commonly referred to as interstitial pancreatitis. Translocation of bacteria from the gut into the systemic circulation may occur as a consequence of gut ischemia secondary to hypovolemia and arteriovenous shunting. Severe pancreatitis may ensue, leading to life-threatening complications such as acute respiratory distress syndrome, renal failure, shock, metabolic derangements, and multi-organ failure. Approximately 20% of patients with acute pancreatitis will have a severe course with a 10% to 30% mortality rate[3]. Mortality related to acute pancreatitis decreased substantially from as high as 25%-30% in the 1970s to current rates since the early 1990s; however, since then, the mortality has remained relatively constant[3,4]. Initial improvement appears to have been related to improvements in intensive care treatment rather than a better understanding of the natural history of disease or improved intervention.

Determining the etiology of acute pancreatitis can be challenging in those who do not give a significant history of alcohol use and in those who do not exhibit obvious gallstone disease. In about 20% to 40% of cases, no defined cause may be found and patients are subsequently labeled as having idiopathic acute pancreatitis[5]. Several causes for pancreatitis may be missed in the initial workup using the conventional imaging techniques of

trans-abdominal ultrasound and/or CT scan and routine laboratory tests. Other modalities, including more advanced imaging techniques and endoscopic procedures, are often considered when working up the cause of unexplained acute pancreatitis. Although used primarily for diagnostic and therapeutic purposes in biliary disorders, endoscopic retrograde cholangiopancreatography (ERCP) has evolved as a diagnostic and therapeutic option in evaluating several pancreatic diseases[6].

CLINICAL USE OF ERCP IN ACUTE PANCREATITIS

When clear clinical, laboratory and imaging evidence for persistent biliary obstruction is present, patients should directly proceed to ERCP. As with all invasive procedures, the risk of procedure-related complications must be weighed against the potential benefit of the procedure. ERCP has a reported complication rate of 5% to 7%, with the main complications including pancreatitis, hemorrhage, perforation, cholangitis, cardiopulmonary complications and, rarely, death. Patient-related factors, such as underlying co-morbidities, age, and need for invasive evaluation, are considerable determinants of complication risk in any endoscopic procedures, especially those that carry a higher possibility for complications such as ERCP. Because of the potential morbidity and mortality associated with ERCP and the improvements in other less-invasive imaging modalities, the role of ERCP has become more well-defined in the diagnosis and treatment acute pancreatitis.

In the 30% of patients who may have no identifiable cause for acute pancreatitis with traditional non-invasive methods, ERCP with empiric biliary sphincterotomy is often performed without a more thorough evaluation for cause[7]. Freeman *et al* evaluated this concern and determined the risk of post-ERCP pancreatitis to be as high as 20% with a 3%-4% risk for severe pancreatitis. Though tempting, empiric sphincterotomy appears to have about an equal chance of causing complications as treating the underlying cause of the acute pancreatitis and, therefore, is not advocated[6,8]. Determining the etiology of acute pancreatitis is important, as it helps direct therapy, limits further unnecessary evaluation, and may improve a patient's long term prognosis. Advanced endoscopic procedures, including ERCP, are emerging as valuable tools in the evaluation of this challenging group. The role of ERCP in the diagnostic and therapeutic evaluation of acute pancreatitis will be discussed in this review.

MICROLITHIASIS

Microlithiasis or biliary sludge as a causative etiology for acute pancreatitis remains controversial and not well understood. Several studies have demonstrated the presence of biliary sludge in as many as 75% of patients with unexplained acute pancreatitis[5]. Microlithiasis is a viscous precipitate containing mucin, cholesterol and calcium bilirubinate which can obstruct the pancreatic duct. Ultrasonography has a sensitivity of only about 55% in detecting microlithiasis and does not allow for analysis of the chemical composition of bile[9]. Bile analysis with microscopic examination is considered the gold standard for diagnosis. Bile can be obtained directly while cannulating the bile duct during ERCP or following CCK stimulation on EGD. ERCP with bile aspiration from the common bile duct (CBD) has a reported sensitivity of 83% in detecting microlithiasis[7]. It is recognized, however, that gallbladder bile is preferred over ductal bile for examination, as transit through the hepatic and common ducts can be too rapid to allow formation of crystals large enough to detect on microscopy. Ko *et al*[9] propose the criteria of 2 or more crystals per 100X field or more than 4 crystals per sample as a positive result. It is also recommended to collect bile samples prior to contrast injection to avoid the formation of "pseudomicrolithiasis" from contrast precipitates. Using a guidewire under fluoroscopy with aspiration to clear the collection catheter prior to obtaining the bile sample can minimize contrast contamination and diminish this artifact. The specimen should be centrifuged immediately and examined under polarized microscopy to evaluate for crystals. If specimens are not examined immediately, crystals can precipitate and cause a false positive result. ERCP should be performed after complete recovery from acute pancreatitis, usually 4 to 6 wk after presentation. If microlithiasis is detected, patients should be considered for cholecystectomy or biliary sphincterotomy depending on surgical risk. In post- cholecystectomy patients, bile analysis need not be performed.

PANCREAS DIVISUM

Pancreas divisum (PD) is the most common congenital anomaly of the pancreas and occurs in approximately 7%-10% of the population; however, less than 5% of those with PD are symptomatic[7]. PD is a small or absent ventral pancreatic duct that fails to fuse with the dorsal duct during embryologic development, resulting in a lack of communication between the ducts (Figure 1A and B). This lack of communication leads to a prominent dorsal duct that drains entirely through the minor papilla. Incomplete PD is the partial communication between the ventral and dorsal ducts; however, incomplete PD functions similar to that of complete PD. The divided drainage routes may result in a relative obstruction of flow through the minor papilla, leading to acute pancreatitis. Some controversy exists about recognizing PD as a causative etiology for pancreatitis. Regardless of the debate, several studies have demonstrated that dorsal duct outflow obstruction can lead to both acute and chronic pancreatitis[10,11]. It has been reported that patients with divisum have an increased prevalence of pancreatitis. Parenchymal changes consistent with chronic pancreatitis isolated to the dorsal pancreas have been observed on autopsy studies[6].

ERCP plays a limited role in diagnosis of PD given the less invasive and reasonably accurate modalities of MRCP and EUS. ERCP can, however, offer a therapeutic option in those who experience recurrent acute pancreatitis. Several studies have shown that endoscopic intervention, including sphincterotomy with or without stenting of the minor

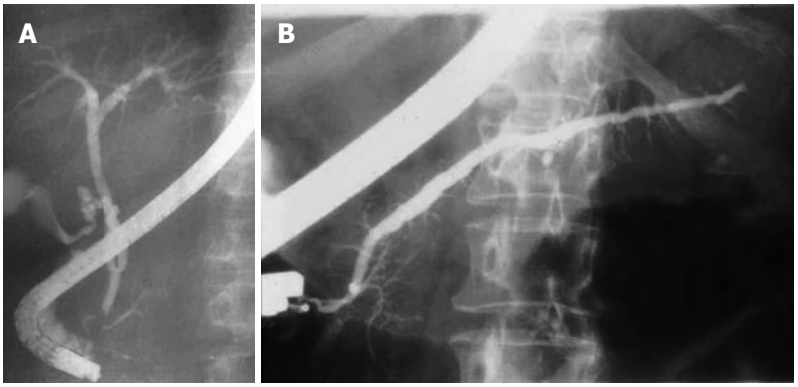

Figure 1 **A**: Pancreas divisum with filling of the small ventral duct; **B**: Pancreas divisum with filling of dorsal duct through the minor orifice.

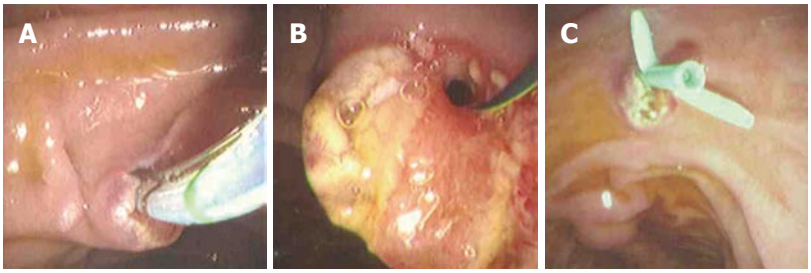

Figure 2 **A**: Sphicterotome performing minor ampulla sphincterotomy; **B**: Guidewire in place after minor sphincterotomy; **C**: Pancreatic stent placement post-sphincterotomy for pancreas divisum.

papilla, has resulted in decreased recurrence rates of acute pancreatitis and improved outcomes in patients with PD (Figure 2A-C)[12-17]. In the only randomized controlled trial evaluating endoscopic therapy in PD, Lans *et al*[18] observed an improvement in 90% of treated patients versus only 11% of controls over a mean of 12 mo follow-up. A retrospective study by Gerke *et al*[19] observed that 60% of patients who underwent minor papillotomy reported immediate improvement, but sustained results were seen in only half of those patients at a mean of 29 mo follow-up. Patients with well-defined bouts of pancreatitis appeared to benefit over those solely with chronic abdominal pain without objective evidence of pancreatitis. It is recognized that pain secondary to PD and chronic abdominal pain with incidentally found PD is difficult to differentiate. In order to distinguish between these two groups, objective criteria such as recurrent pain with ductal dilatation and elevations in pancreatic enzymes can be useful. Secretin stimulation imaging using either ultrasonography or MRI has proven promising as these modalities can give clues to the functionality of the pancreas. The study by Gerke *et al* underscores the importance of defining patients who may benefit from endoscopic therapy and reveals the difficulty in achieving long-term results in patients with PD.

The role of using pancreatic stents in PD is limited to the prevention of post-ERCP pancreatitis and has demonstrated little efficacy in treating PD in comparison to sphincterotomy. The long-term use of pancreatic stents in PD may induce duct strictures or irregularities. Most experts recommend only the short-term use of pancreatic stents (less than 2 wk) in patients without pre-existing duct strictures. Size 3 or 4 French, non-phlanged stents, without an internal flap are recommended to decrease the risk of post-ERCP pancreatitis (Figure 3). These stents can pass spontaneously, therefore, obviating the need for a repeat procedure for stent removal. Note that the use of size 3 French stents requires a separate, smaller guidewire which may increase the overall cost of the procedure.

SPHINCTER OF ODDI DYSFUNCTION

Sphincter of Oddi dysfunction (SOD) as an etiology for acute pancreatitis has been questioned by many due to the lack of concrete pathologic findings[20]. SOD refers to an abnormality in sphincter of Oddi contractility resulting in intermittent biliary and pancreatic duct obstruction. It has been estimated that SOD accounts for approximately 1/3rd of those with recurrent unexplained pancreatitis.

Management depends upon the classification of SOD. A classification system similar to the Milwaukee Biliary Group Classification has been developed for pancreatic SOD (Table 1): type Ⅰ pancreatic SOD includes recurrent pancreatitis or pain suspected to be of pancreatic origin with elevated amylase and/or lipase up to 1.5 times upper limit of normal, a dilated pancreatic duct greater than 6 mm in the head and more than 5 mm in the body, and delayed emptying of greater than 9 min of the pancreatic duct. type Ⅱ SOD includes the presence of presumed pancreatic pain plus at least one additional factor defining type Ⅰ. type Ⅲ is defined by pain alone. Some experts have suggested that due to the high incidence of stone disease, patients suspected to have SOD with an intact gallbladder should undergo cholecystectomy as initial therapy before evaluation for sphincter dysfunction due to less risk involved in surgery compared with manometry. Our approach in patients with intact gallbladder, but no stone disease on standard imaging, is to perform endoscopic ultrasound (EUS) looking for undetected stones or biliary sludge. If EUS is unremarkable, then ERCP with bile analysis is

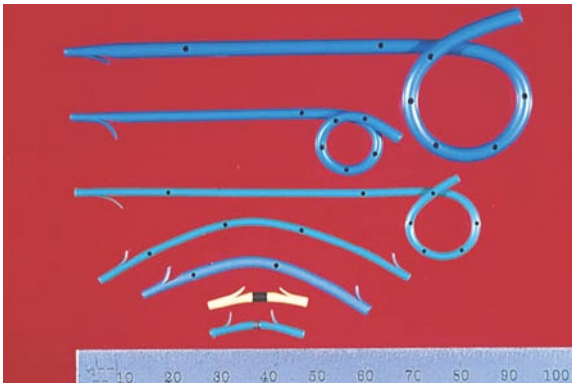

Figure 3 Various designs of pancreatic stents (with permission from Cook Endoscopy).

performed to evaluate for microlithiasis. If microlithiasis is not detected, we will proceed with sphincter of Oddi manometry during the same exam. In those who have previously had a cholecystectomy, dual sphincterotomy becomes the recommended treatment modality. A study by Gelrud *et al*[21] demonstrated significantly better outcomes when dual sphincterotomy was performed over a biliary sphincterotomy alone.

Management of types Ⅱ and Ⅲ disease has posed more of a treatment challenge to the clinician. Patients who meet type Ⅱ classification criteria should undergo sphincter of Oddi manometry (SOM). A resting basal pressure of > 40 mm Hg is the best predictor of response to endoscopic sphincterotomy, with up to 90% demonstrating clinical benefit in 4 years follow-up[20]. Studies advocate the measurement of both biliary and pancreatic sphincter pressures during ERCP, as differences in pressure may be detected if either duct is measured alone[22-25]. There is no role for ERCP without manometry in the evaluation of type Ⅱ SOD patients.

Traditional teaching has been that performing SOM increases the risk for pancreatitis[26]. However, more recently, it appears that performing any ERCP (with or without manometry) in the subset of patients at highest risk for SOD (typically young women with unexplained recurrent abdominal pain, normal anatomy, and normal serum bilirubin) increases the risk for pancreatitis[8,27,28]. Several randomized trials have proven a decreased risk of post-ERCP pancreatitis in this group of patients by performing prophylactic stent placement[29,30]. The NIH has recommended that diagnostic and therapeutic procedures in this select group of patients should be performed ONLY by endoscopist possessing expertise in this particular area because of the high rate of severe complications in this young, otherwise healthy population[20]. Because of this thought, the diagnosis and management of type Ⅲ disease is the most difficult. Invasive procedures should be delayed or avoided if possible. Trials of anticholinergics, antidepressants, nonspecific pain relievers and/or calcium channel blockers should precede invasive approaches. Diagnostic ERCP without manometry has no role in the assessment of type Ⅲ pancreatic SOD patients[20].

OCCULT TUMORS OF THE PANCREAS AND BILIARY TRACT

Approximately 30 000 new cases of pancreatic cancer and 7000 biliary tract cancers are diagnosed annually in the United States. Prognosis remains dismal with less than 20% survival at 1 year and a 5 years mortality rate of > 95%[31]. Infrequently, tumors of the pancreas and biliary tree may present as acute pancreatitis. ERCP has proven to be valuable in the diagnosis and treatment of ampullary tumors and intraductal papillary mucinous tumors (IPMT) of the pancreas, both conditions which may present with acute pancreatitis due to obstruction from the pancreatic duct. ERCP may be the best means for direct visualization of the ampullary and periampullary region and offers the capability of sampling through biopsy. In non-operative patients with obstructing ampullary tumors, palliation or possibly cure can be achieved with endoscopic snare ampullectomy and ablative thermal therapy. Post-procedure stent placement in this clinical scenario has been demonstrated to reduce the risk of post-procedure pancreatitis[6]. IPMT can be diagnosed on ERCP with the classic "fish eye" appearance of the dilated ampulla and mucin extruding from the orifice (Figure 4). In non-operative patients with IPMT, ERCP with stent placement and/or sphincterotomy may offer palliation by decreasing the risk of acute recurrent pancreatitis by minimizing mucin impaction in the pancreatic duct.

Peroral pancreatoscopy (PPS) is useful in cases of suspected IPMT by not only diagnosing but also localizing the tumor for planning surgical resection. PPS may also provide tissue sampling for histologic diagnosis[32].

Table 1 Sphincter of Oddi Dysfunction

Type Ⅰ
1 Typical biliary-pancreatic pain
2 Liver chemistries (total bilirubin, alkaline phosphatase, transaminases) ⩾ 1.5-2X ULN and/or pancreatic chemistries (amylase and/or lipase) ⩾ 1.5-2X ULN
3 Dilated common bile duct (⩾ 12 mm) or pancreatic duct (head ⩾ 6 mm, body ⩾ 5 mm) diameter
4 Prolonged biliary drainage (> 45 min) with patient in supine position or pancreatic drainage (> 9 min) with patient in the prone position
Type Ⅱ
1 Typical biliary-pancreatic pain
2 Positive findings for one or two items (2, 3, or 4) from type Ⅰ
Type Ⅲ
1 Typical biliary-pancreatic pain and no other abnormalities

Modified from Sherman *et al*[25].

GALLSTONES

Gallstones account for nearly half of the cases of acute pancreatitis in the Western world. More than 70% of patients will spontaneously pass the culprit stone into the duodenum, however, approximately 3% to 7% may go on to develop acute pancreatitis[33]. Because differentiating patients who may have an uncomplicated course due to a transiently impacted gallstone from those who may

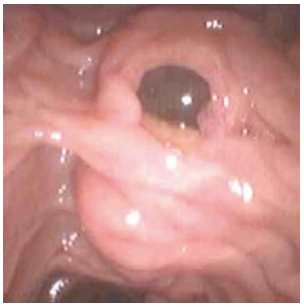

Figure 4 Classic "fish eye" appearance of IPMT with mucin draining from minor ampulla.

progress on to severe acute pancreatitis with necrosis and sepsis is difficult, several studies have evaluated scoring systems and variables to predict severity[34-36]. Urgent endoscopy is generally reserved for patients who fail to demonstrate liver enzyme improvement within 24 to 48 h of admission, especially in total bilirubin by hospital d 2[34]; those demonstrating persistent choledocholithiasis on imaging; and those with clinical cholangitis. Fan *et al*[37] evaluated the role of early ERCP in patients with acute biliary pancreatitis prior to the onset of complications, regardless of mild or severe presentation. Within 24 h of presentation, patients were randomized to early ERCP versus conservative treatment with selective ERCP. A significant reduction in progression to biliary sepsis was seen in the early ERCP patients. Interestingly, however, the incidence of local and systemic complications was not significantly different, which suggests that removal of the impacted stone may not reverse the damage already occurring in the pancreas during the first hours or days of the illness. Other studies have advocated early intervention within 72 h after admission if persistent CBD stones were suspected[38,39].

Patients who recover from gallstone pancreatitis carry a 29% to 67% risk of recurrent pancreatitis if subsequent cholecystectomy and/or sphincterotomy are not performed[6,40]. In mild to moderate gallstone pancreatitis, ERCP is rarely required before cholecystectomy unless cholangitis or clear evidence of persistent choledocholithiasis by imaging and laboratory data is observed. Chang *et al*[34] evaluated patients with acute gallstone pancreatitis who were suspected of persistent choledocholithiasis. Patients were randomized to either pre-operative ERCP or selective post-operative ERCP if choledocholithiasis was found intraoperatively. Hospital stay was significantly longer in the routine pre-operative ERCP group (11.7 d *vs* 9.0 d). In the post-operative group, ERCP was necessary in only 24% of patients, suggesting that the diagnostic and therapeutic yield of pre-operative ERCP is low. These findings are consistent with the NIH consensus statement recommending that patients suspected of having choledocholithiasis should undergo an operative cholangiogram at the time of cholecystectomy. Operative cholangiogram is efficient and preferable when surgical proficiency in this technique is available. Otherwise, post-operative ERCP is indicated for patients who demonstrate retained stones. In patients who have had a prior cholecystectomy and have a low probability of common bile duct stones, diagnostic evaluation for choledocholithiasis should be performed with less-invasive modalities including MRCP or EUS. In the clinical scenario where the potential for retained common bile duct stones is substantial, ERCP and, when indicated, sphincterotomy with stone removal is the preferred diagnostic and therapeutic option[20]. ERCP with sphincterotomy is the preferred therapeutic modality if cholangitis from retained common bile duct stones is present. Patients should receive close medical care and treatment with IV fluid resuscitation, hemodynamic monitoring, and intravenous antibiotic therapy. Patients who fail to improve should undergo ERCP with sphincterotomy as soon as possible. Those who do improve still require urgent ERCP with sphincterotomy, usually within 24 h, to relieve the obstruction[20].

Recurrence of pancreatitis after ERCP with sphincterotomy for gallstone pancreatitis is rare. Cholecystectomy versus endoscopic sphincterotomy for the treatment of recurrent gallstone pancreatitis remains a controversial topic. Several experts advocate that cholecystectomy should only be considered if there are overt manifestations of gallbladder disease (e.g., biliary pain, cholecystitis, cystic duct obstruction), but not for prevention of recurrent gallstone pancreatitis. Studies have demonstrated that ERCP can be an effective therapeutic option for prevention of recurrent gallstone pancreatitis[41]. Siegel *et al*[42] demonstrate that ERCP with sphincterotomy can be performed safely in both the elective and urgent setting in patients who are otherwise not ideal operative candidates, such as the aged or younger patients at risk for surgical complications. Our approach is to proceed with cholecystectomy if the patient is a good surgical candidate.

PANCREATIC PSEUDOCYSTS AND FLUID COLLECTIONS

Pancreatic pseudocysts occur mainly as a result of acute pancreatitis, pancreatic trauma or chronic pancreatitis. Fluid usually contains a high concentration of pancreatic enzymes and variable amount of tissue debris. Most pseudocysts are sterile. ERCP has a reported success rate of 65%-95% in treatment of pancreatic pseudocysts, with a complication rate of 10%-35%. Drainage of fluid collections is generally reserved for a later date, usually 4-6 wk after the acute pancreatitis episode resolves.

PANCREATIC DUCT INJURY

Pancreatic duct disruptions may result from acute and chronic pancreatitis, or they may be the primary cause for pancreatitis in cases of trauma or surgical injury. ERCP can be successful in detecting the presence of contrast extravasation from the duct, localizing the suspected site of injury, and treating the leak or fistula with stent or drain placement.

Approximately 37%-67% of patients with acute pancreatitis have pancreatic duct injury, suggesting that acute duct injury can be a relatively common finding[43,44]. Lau *et al*[43] observed that the presence of a leak was associated with a higher incidence of necrosis and prolonged length of stay. ERCP in this patient population was determined to be safe and not associated with

increased mortality, prolonged hospital stay or need for necrosectomy provided that pancreatic duct leaks were detected and immediately treated.

ERCP to evaluate for pancreatic duct disruption in acute pancreatitis is controversial and should be reserved for investigational studies. A multidisciplinary approach is advocated when considering pancreatic stenting in the setting of acute necrosis, as the procedure carries a risk of introducing infection into an otherwise sterile environment. In patients with evidence of pancreatic duct injury or leak who are not responding to conservative treatment, ERCP should be considered.

The use of ERCP in the treatment of acute pancreatitis from traumatic pancreatic duct injury has also been evaluated in both the adult and pediatric patient population. Several studies have demonstrated that ERCP with transpapillary stent placement is an effective technique in closing pancreatic duct disruption[45-47]. Successful therapy, however, appears to be associated with positioning of the stent to bridge the disruption and leak, not simply across the papilla as in biliary leaks[48,49]. Several studies in children have also reported successful results in treating traumatic duct injury, however the authors call attention to the risk of iatrogenic ductitis when stenting smaller pancreatic ducts, especially those in children[50,51].

UNUSUAL CAUSES OF ACUTE PANCREATITIS

Type Ⅲ choledochal cysts are dilations of the joined portion of the pancreaticobiliary ducts. These cysts can be large enough to obstruct the pancreatic duct, which may result in recurrent acute pancreatitis[52,53]. Biliary sphincterotomy has been suggested as treatment; however, some patients may require a dual sphincterotomy for long term benefit.

Annular pancreas, anomalous pancreaticobiliary junction, and pancreatic intraductal parasites have all been reported as causes for acute and recurrent acute pancreatitis. ERCP can occasionally offer benefit in treatment of these rare conditions[6].

CONCLUSION

ERCP is a useful tool in the evaluation and management of acute pancreatitis. The main role of ERCP in acute pancreatitis is the diagnosis and treatment of biliary tract stone disease and other potential causes of pancreatic duct obstruction including sphincter dysfunction or anomalies such as pancreas divisum. With the advent of less-invasive and safer diagnostic modalities, ERCP is appropriately becoming a therapeutic tool in the management of acute pancreatitis and its complications.

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TOPIC HIGHLIGHT

Michael F Byrne, MD, *Series Editor*

Utility of endoscopic ultrasound in pancreatitis: A review

Maged K Rizk, Henning Gerke

Maged K Rizk, Henning Gerke, University of Iowa Hospitals and Clinics, Division of Gastroenterology and Hepatology, Department of Internal Medicine, 200 Hawkins Drive, Iowa City, IA 52242, United States
Correspondence to: Henning Gerke, MD, Assistant Professor, Department of Internal Medicine, Division of Gastroenterology and Hepatology, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA 52242, United States. henning-gerke@uiowa.edu
Telephone: +1-319-3562132 Fax: +1-319-3536399
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Abstract

The close proximity of the endoscopic ultrasound probe to the pancreas results in superior spatial resolution compared to CT scan and MRI. In addition, endoscopic ultrasound (EUS) is a minimally invasive procedure that does not share the relatively high complication rate of ERCP. Due to these advantages, EUS has evolved into an important technique to assess pancreatobiliary disease. This review will discuss the role of EUS in patients with pancreatitis. The indications can be divided into acute pancreatitis and chronic pancreatitis. In acute pancreatitis, EUS is used to determine the etiology; in suspected chronic pancreatitis it is helpful to establish the diagnosis. Lastly, this review will discuss biliary pancreatitis with suspicion for persistent choledocholithiasis.

Key words: Idiopathic pancreatitis; Acute pancreatitis; Chronic pancreatitis; Endoscopic ultrasound; Endosonography; Pancreas divisum; Cholelithiasis; Microlithiasis; Choledocholithiasis; Biliary pancreatitis

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INTRODUCTION

Overlying intestinal gas and the retroperitoneal location of the pancreas distant from the abdominal wall can impair the visualization of this organ with trans-abdominal ultrasound. This problem has been overcome by integrating the ultrasound probe into an endoscope in order to place it directly into the gastric and duodenal lumen. The close proximity of the endoscopic ultrasound probe to the pancreas results in high spatial resolution that is superior to that of Computer Tomography (CT) and magnetic resonance imaging (MRI). In addition, endoscopic ultrasound (EUS) is a minimally invasive procedure that does not share the relatively high complication rate of endoscopic retrograde cholangiopancreatography (ERCP). Due to these advantages, EUS has evolved into an important technique to assess pancreatobiliary disease.

This review will discuss the role of EUS in patients with pancreatitis. The indications can be divided into acute pancreatitis and chronic pancreatitis. In acute pancreatitis, EUS is used to determine the etiology; in suspected chronic pancreatitis it is helpful to establish the diagnosis. Another indication that will be discussed is biliary pancreatitis with suspicion for persistent choledocholithiasis.

ACUTE IDIOPHATHIC PANCREATITIS

The diagnosis of acute idiopathic pancreatitis (AIP) is applied when an etiology cannot be determined after the initial evaluation that includes a thorough history and physical exam, laboratory evaluation and abdominal ultrasound or CT[1-4]. In-depth evaluation of AIP using EUS often yields the diagnosis of microlithiasis, pancreatic divisum, chronic pancreatitis[1,5-7] or even neoplasm[1,4].

Occult gallstones and microlithiasis

A substantial number of patients with AIP and unexplained biliary pain turn out to have biliary sludge or small gallstones that have gone undetected by abdominal ultrasound (US) or CT. The term 'biliary microlithiasis' was coined to describe gallstones of < 3 mm in diameter[8-10]. Although sonographic characteristics of cholelithiasis do not differ between EUS and trans-abdominal ultrasound, EUS is more sensitive in detecting gallstones[11] due to the proximity of the endoscope tip to the gallbladder. Small gallbladder stones present as bright floating foci. Larger stones have posterior shadowing. Sludge presents as hyperechoic content within the gallbladder or bile duct (Figures 1 and 2).

The reported incidence of occult gallstones in patients with AIP varies widely. It ranges from 10%-73%[12-15]. Gallstones remain the most common cause of pancreatitis in patients with intact gallbladder. Therefore, it is

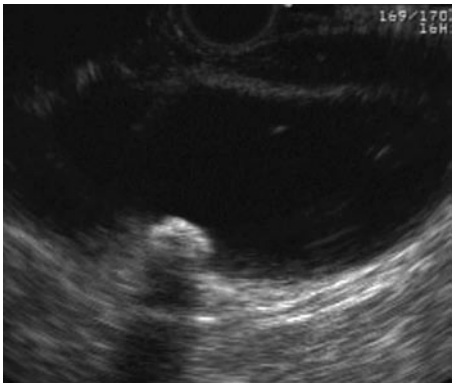

Figure 1 Linear EUS of gallbladder with shadowing stone.

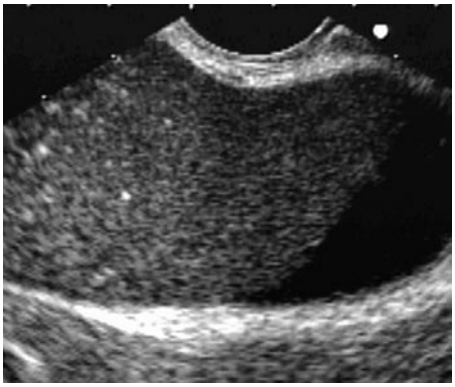

Figure 2 Linear EUS of gallbladder with sludge, and bright shadowing foci representing small stones.

commonly believed that the finding of microlithiasis explains the etiology of the pancreatitis. This has recently been challenged. In a study by Garg *et al*. Seventy-five patients with AIP were studied with duodenal bile microscopy and EUS. Initially, the cause of the recurrent pancreatitis was attributed to biliary microlithiasis in 10 of 75 patients. Eight of these 10 patients underwent cholecystectomy or endoscopic sphincterotomy yet continued to have recurrent pancreatitis flares[16] which implies that gallstones or biliary crystals were innocent bystanders in these patients. In contrast, other studies have demonstrated response to cholecystectomy[5,17], sphincterotomy[11] or ursodeoxycholic acid (UDCA)[10] in patients with microlithiasis suggesting a causal relationship.

Liu *et al* prospectively evaluated 89 consecutive patients who presented with symptoms of acute pancreatitis with trans-abdominal ultrasound, CT, or both. ERCP was performed in all patients with confirmed or suspected biliary pancreatitis. EUS was performed in patients suspected of having idiopathic pancreatitis. Of the 18 patients classified as idiopathic pancreatitis who underwent EUS, 14 had stones that were between 1 and 9 mm in size which was confirmed by cholecystectomy. Three had concomitant choledocholithiasis confirmed by ERCP[17].

Another study retrospectively evaluated 31 patients with AIP who underwent EUS 2-3 wk after resolution of symptoms[5]. Five of 31 patients had microlithiasis diagnosed by EUS (n = 3), or by bile microscopy after EUS (n = 2). All 5 patients underwent cholecystectomy and remained asymptomatic during the follow-up period. Sludge was found on pathology examination in all 5 gallbladders. Gallstones or sludge were not diagnosed in any of the other 26 subjects during the follow-up period.

In summary, EUS is an effective modality in diagnosing microlithiasis and may strengthen the indication for a subsequent intervention. Treatment with cholecystectomy, endoscopic sphincterotomy or ursodeoxycholic acid may reduce recurrent attacks of pancreatitis[10,11]. However, it remains debatable how intensively we have to search for occult gallstones. Statistically, gallstones remain by far the likeliest cause of unexplained recurrent pancreatitis in patients with intact gallbladders. The morbidity of laparoscopic cholecystectomy is very low, and one could argue that this procedure is justified regardless of the findings of cross sectional imaging.

Pancreas divisum

Pancreas divisum is a common congenital malformation. The prevalence is estimated at 5%-10% in a Western population[18]. This abnormality is characterized by lack of connection between the dorsal and ventral pancreatic ducts due to incomplete fusion of the pancreatic buds during embryologic development. As a result, the ventral duct drains only a small portion of the pancreas *via* the major papilla, whereas the dorsal pancreatic duct drains the majority of the pancreas *via* the minor papilla. The small size of the minor papilla in relation to the drainage volume may lead to relative outflow obstruction. Since only a minority of patients with pancreas divisum becomes symptomatic, it has been suggested that symptomatic disease requires additional factors leading to minor papilla stenosis. Symptomatic patients present with recurrent acute pancreatitis, chronic pancreatitis, or chronic abdominal pain without evidence of pancreatitis. Pancreas divisum has been implicated in as much as 20% of patients with AIP[12]. Patients with discrete episodes of acute pancreatitis commonly improve after ERCP with minor papillotomy, whereas the results are less favorable for those with chronic pancreatitis or chronic abdominal pain[19] .

ERCP is the gold standard for the diagnosis of pancreas divisum but poses a risk of post procedure pancreatitis. Small series suggest that EUS enables a fairly reliable diagnosis of pancreas divisum and may therefore present an alternative to ERCP with minimal complication rate[5,7,20,21]. Different EUS-criteria have been used: Bhutani *et al* suggest that the absence of a "stack sign" may be useful in determining the diagnosis. The stack sign is obtained by positioning a radial echoendoscope in the long position with the transducer in the duodenal bulb. The balloon is then inflated and advanced snugly into the apex of the bulb. From this position, the bile duct and the pancreatic duct can be seen running parallel through the pancreatic head. In six patients with known pancreas divisum that underwent EUS, the stack sign was found in only two patients. Of the two patients with presence of a stack sign, one had a ventral duct that was markedly dilated, and the other patient had an unusually large ventral pancreas[20]. Tandon *et al* used different sonographic criteria. The authors required direct visualization of the dorsal duct coursing to the duodenal wall, and excluded patients with a sonographically visible ventral pancreatic duct. The authors feel that their criteria will exclude some

cases of pancreas divisum and many cases of "incomplete pancreas divisum," but may be more specific as compared to the absence of a stack sign[5]. Lai *et al* suggests that evaluation using a linear-array echoendoscope is possible. The main pancreatic duct can be followed continuously from the major papilla into the pancreatic body. The duct can be seen crossing a sonographic border between the ventral and dorsal pancreas. Absence of this feature suggests pancreas divisum. In the retrospective study, of the 78% who had adequate visualization of the pancreatic duct, sensitivity, specificity, positive and negative predictive values for EUS were 95%, 97%, 86%, and 99%, respectively[21].

Occult neoplasm

It has been estimated that pancreatic neoplasms cause pancreatitis at some point in the disease course in up to 7 percent of patients[22]; however, they are a rare differential diagnosis in patients with AIP.

Mujica *et al* surveyed 19 physicians regarding 45 patients who presented with acute pancreatitis prior to a diagnosis of a neoplasm. The patients had a mean number of 2 episodes of acute pancreatitis prior to the diagnosis of neoplasm. The mean time to diagnosis of the neoplasm after the initial episode was 34 wk. The majority of patients were diagnosed using conventional cross-sectional imaging, whereas only 3 patients in the series were diagnosed using EUS[22].

Albeit rare, it has been suggested that pancreatic malignancy should be suspected in patients with unexplained pancreatitis who are older than 40 years of age[23]. EUS is superior to CT in detecting small pancreatic neoplasms[24,25], however, inflammatory changes during a pancreatitis flare may decrease the image quality. Therefore, cross-sectional imaging and/or EUS should be repeated after the resolution of the acute attack.

Single episode of idiopathic pancreatitis

The utility of an evaluation with EUS after a single episode of unexplained pancreatitis is not well studied and remains unclear[4]. In a small series by Tandon *et al*, EUS found an etiology in 7 of 14 patients with a single episode of idiopathic pancreatitis (3 microlithiasis, 1 pancreas divisum, 3 alcoholic chronic pancreatitis). The diagnosis changed in only 1 patient[23] during the follow-up period. A series reported by Yusoff *et al* included 201 patients with a single episode of acute pancreatitis. A presumptive diagnosis was made after EUS in 31%; chronic pancreatitis and sludge were the most common diagnoses in those with a gallbladder, whereas chronic pancreatitis and pancreatic divisum were the most prevalent diagnoses in patients who had a prior cholecystectomy[6].

Although these studies suggest a high yield of EUS in patients with a single episode of unexplained pancreatitis, some skepticism remains. Only 20%-50% of patients will have recurrent symptoms[26] following the initial attack. Furthermore, it is difficult to be sure about the causal relationship of an abnormal EUS finding after a single episode of pancreatitis. Pancreas divisum, for example, is common in the general population, and may be a coincidental finding rather than the cause of the pancreatitis. Even microlithiasis may be a harmless bystander[16]. As discussed in detail in a later paragraph, the diagnosis of chronic pancreatitis with EUS is problematic due to lack of specificity in early stages. In our opinion, further studies are necessary before advocating EUS for every patient after a single episode of idiopathic pancreatitis.

Table 1 EUS criteria of chronic pancreatitis

Parenchymal criteria	Pancreatic ductal criteria
Hyperechoic foci	Dilation (4 mm in head, 3 mm in body, 2 mm in tail)
Hyperechoic strands	Irregularity
Lobularity	Hyperechoic duct margins
Heterogeneity	Visible branch ducts
Shadowing calcifications	Intraductal stones
Cysts	

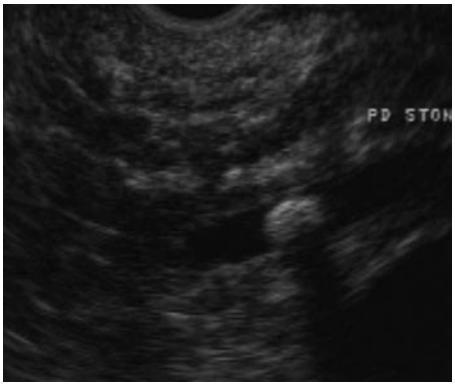

Figure 3 Linear EUS showing a shadowing stone within the pancreatic duct (PD STONE).

CHRONIC PANCREATITIS

The diagnosis of chronic pancreatitis (CP) can be challenging. The normal pancreas has a homogeneous fine granular echo-pattern (salt and pepper appearance), with a thin and regular main pancreatic duct. Certain sonographic changes can be observed in patients with CP. In an attempt to develop diagnostic scores for the EUS-diagnosis of chronic pancreatitis, "EUS criteria" have been developed. These were first described by Jones *et al*[27], and later refined by Wiersema *et al*[28]. The criteria can be divided into pancreatic duct findings and parenchymal findings. Parenchymal findings include hyperechoic foci, hyperechoic strands, lobularity, heterogeneity, shadowing calcifications, and cysts. Pancreatic duct findings include dilation (> 4 mm in the head, > 3 mm in the body, > 2 mm in the tail), irregularity, hyperechoic duct margins, and visible side-branches (Table 1, Figures 3-6). Multiple studies have evaluated the ability of EUS to diagnose CP using the above criteria. In a prospective, blinded study by Sahai *et al*, 126 patients who were admitted for abdominal pain underwent ERCP followed by EUS performed by a blinded operator. ERCP diagnosis of CP was based on Cambridge Criteria. EUS sensitivity was uniformly greater than 85% when the diagnosis of CP was based on the

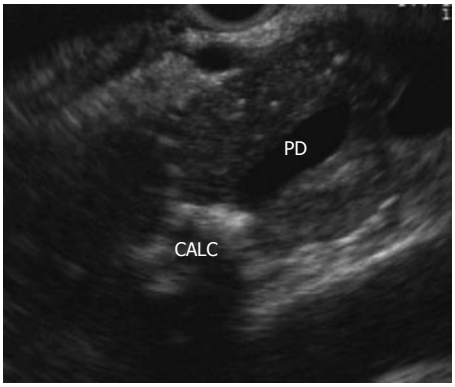

Figure 4 Linear trans-gastric EUS of the pancreas showing parenchymal calcifications (CALC) causing dilatation of the upstream pancreatic duct (PD).

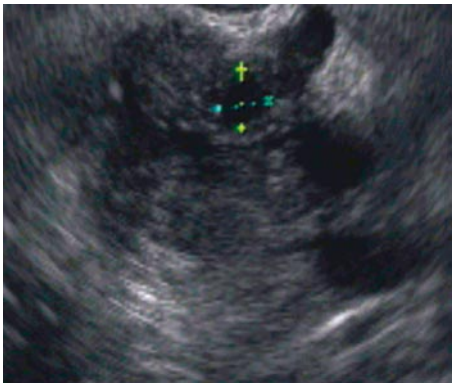

Figure 5 Linear trans-gastric EUS showing a small pancreatic cyst (labeled with measurement markers).

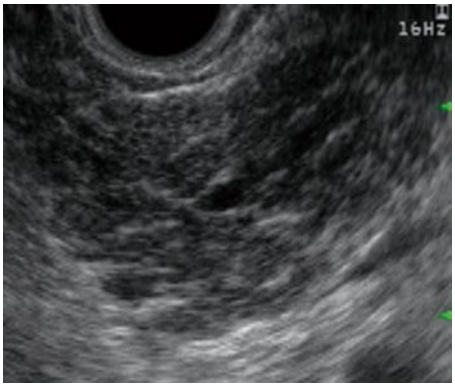

Figure 6 Linear EUS of pancreatic body with echogenic strands and lobularity.

presence of fewer than three criteria, but the specificity was less than 60%. Specificity increased as the number of criteria increased and was greater than 85% when more than five criteria were used. "Moderate to severe chronic pancreatitis" was unlikely (NPV > 85%) when fewer than three criteria were present[29]. When criteria that can easily be detected by other imaging methods (ductal dilation, calcification, and cysts) were excluded, the number of parenchymal EUS criteria remained an independent predictor of CP[29].

There are nuances that need to be considered when using the above score. Firstly, the role of ERCP as a diagnostic "gold-standard" is debatable[30]. Thus, it is difficult to determine whether EUS is over-diagnosing pancreatic disease based on minimal changes or whether ERCP is a false negative in those with abnormal EUS findings but normal ERCP. In a study by Kahl *et al*[31], 32 patients with abnormal EUS but normal initial pancreatogram developed findings of CP on repeat ERCP after a median follow-up of 18 mo suggesting that EUS findings may precede ERCP findings. The sensitivity to diagnose chronic pancreatitis was 100% for EUS, but only 81% for ERCP.

Another concern when using an EUS scoring system to diagnose CP is that not all criteria may be equally important. For example, the presence of intraductal calcifications or parenchymal calcifications alone may be diagnostic of CP even in the absence of other criteria[30]. Age related changes in the pancreas may also affect the diagnostic threshold. The pancreatic duct becomes progressively wider with a hyperechoic wall with increased age. Another aspect to consider is interobserver variability of different criteria. Wiersema *et al*[28] found excellent interobserver agreement among 3 experienced endosonographers reading individual criteria of CP. There was 88% interobserver agreement on presence of echogenic foci, 94% agreement on focally reduced echogenicity, 94% agreement on lobular gland pattern, 83% agreement on the main pancreatic duct echogenicity, and 94% agreement on main pancreatic duct irregularity. On the contrary, Wallace *et al*[32] could not confirm these optimistic results. EUS-exams on 33 patients with suspected CP and 12 controls without suspected CP were videotaped by 3 experienced endosonographers. Eleven expert endosonographers, who were blinded to clinical information, independently evaluated the examinations for the presence of CP and were asked to rank the importance of individual EUS features. There was moderately good interobserver agreement in the final diagnosis of CP (Kappa = 0.45).

Interobserver agreement was good for the individual criteria "ductal dilation" and "lobularity" but was poor for the other 7 criteria. The presence of stones was regarded as the most predictive feature of CP by all endosonographers, followed by visible side branches, cysts, lobularity, irregular main pancreatic duct, hyperechoic strands, main pancreatic duct dilation and main duct hyperechoic margins[32].

In our opinion, the early diagnosis of CP remains problematic due to lack of specificity and the presence of interobserver variability. The overall interpretation of the experienced endosonographer may be more valuable than a diagnosis based on a scoring system.

Only a few studies have evaluated the utility of biopsy in addition to EUS for the diagnosis of CP. One small study suggested that fine needle aspiration may improve the negative predictive value but not the specificity of EUS[33], however this study was limited by the small number of patients without chronic pancreatitis. Out of 37 patients, 31 had chronic pancreatitis. Only 4 patients had normal EUS findings, 3 without and one with chronic pancreatitis (negative predictive value of 75%). The negative predictive value was improved to 100% by FNA-cytology. In our opinion, it is difficult to draw conclusions based on such small numbers. Another study found that EUS-guided core biopsies with a Trucut needle was poor at diagnosing CP[34].

In conclusion, current data do not support a role of EUS-guided biopsies in the diagnosis of CP. In addition

to their questionable diagnostic value, pancreatic biopsies carry a potential risk of post-procedure pancreatitis.

CP makes the detection of pancreatic cancer more difficult. In a series of 282 patients with pancreatic mass (210 with adenocarcinoma), a lower sensitivity for EUS-FNA was observed in patients with CP (more than 4 EUS-criteria) than in those without CP (73.9% *vs* 91.3%). Patients with CP required more EUS-FNA passes to establish a diagnosis versus those without CP (5 *vs* 2)[35].

In summary, the diagnosis of CP remains challenging. EUS criteria have been established. Although these criteria are highly sensitive, they lack specificity in early stages. EUS is accurate in ruling out CP if no pancreatic abnormalities are found and in diagnosing CP if multiple criteria are present. However, a wide grey zone remains for patients with minimal to moderate findings.

CP decreases the sensitivity of EUS-FNA in the evaluation of pancreatic masses.

BILIARY PANCREATITIS AND CHOLEDOCHOLITHIASIS

In most patients with biliary pancreatitis, the causal gallstone has already passed. This makes it difficult to identify those patients in whom ERCP with sphincterotomy may be beneficial. In this context, EUS may provide a minimally invasive modality to diagnose or exclude choledocholithiasis. A review of five studies by Verma *et al* evaluating the efficacy of different modalities in diagnosing choledocholithiasis found an aggregated sensitivity of EUS of 0.93, a specificity of 0.96, a positive predictive value of 0.93, and a negative predictive value of 0.96. There was no statistical difference between MRCP and EUS[36]. In a study by Lui *et al*[37], 100 patients admitted for acute pancreatitis were evaluated with trans-abdominal ultrasound, EUS, and ERCP. EUS was found to be as sensitive as ERCP in the detection of choledocholithiasis, but with a lower complication rate.

Arguedas *et al* proposed a decision analysis model in evaluating biliary pancreatitis. Cost-effectiveness of strategies involving observation, intraoperative cholangiography, EUS, MRCP, and ERCP was evaluated. The results demonstrated that the choice of strategy is strongly influenced by the pretest probability of choledocholithiasis. If cost-minimization is the goal, observation with intraoperative cholangiography at the time of cholecystectomy is preferred in patients considered at "low risk" for choledocholithiasis. EUS is cost effective in patients at "intermediate risk" and ERCP is the preferable strategy in patients at "high-risk". There was no utility for MRCP in this model, as EUS was less costly[38]. Scheiman *et al*[39], also suggested that there is no role for MRCP for biliary pancreatitis in centers where EUS is available.

Sugiyama *et al* prospectively evaluated 35 patients with suspected acute biliary pancreatitis. All patients underwent trans-abdominal ultrasound, CT, EUS, and ERCP. The severity of pancreatitis was graded using APACHE Ⅱ scores. EUS and ERCP were significantly more sensitive in the detection of CBD stones than trans-abdominal ultrasound and CT. ERCP and EUS were equivalent in CBD stone detection. Based on the severity of the pancreatitis, 20 of 35 ERCP were determined to be potentially avoidable[38].

In summary, EUS is both sensitive and specific in the detection of common bile duct stones and has a considerably lower complication rate than ERCP. While patients with high likelihood of cholelithiasis should undergo ERCP directly, EUS may enable selective use of ERCP in those with intermediate likelihood[1,3,7,10,38,39].

CONCLUSION

EUS is helpful in the evaluating patients with AIP and in diagnosing CP. In patients with AIP, EUS enables the diagnosis of occult cholelithiasis, pancreas divisum, chronic pancreatitis or an occult neoplasm. While EUS may be more sensitive than ERCP in diagnosing CP, the specificity is limited in early stages. In biliary pancreatitis, EUS allows accurate detection of common bile duct stones and can be used to select patients who will benefit from ERCP.

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TOPIC HIGHLIGHT

Michael F Byrne, MD, *Series Editor*

Autoimmune pancreatitis: A review

Iman Zandieh, Michael F Byrne

Iman Zandieh, Michael F Byrne, Division of Gastroenterology, Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada
Correspondence to: Dr. Michael F Byrne, Vancouver General Hospital, Division of Gastroenterology, 5th Floor-2775 Laurel Street, Vancouver, British Columbia, Canada, V5Z 1M9, Canada. michael.byrne@vch.ca
Telephone: +1-604-8755640 Fax: +1-604-8755447
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Abstract

Autoimmune pancreatitis has emerged over the last 40 years from a proposed concept to a well established and recognized entity. As an efficient mimicker of pancreatic carcinoma, its early and appropriate recognition are crucial. With mounting understanding of its pathogenesis and natural history, significant advances have been made in the diagnosis of autoimmune pancreatitis. The characteristic laboratory features and imaging seen in autoimmune pancreatitis are reviewed along with some of the proposed diagnostic criteria and treatment algorithms.

Key words: Autoimmune pancreatitis; Chronic pancreatitis; Idiopathic pancreatitis; Sclerosing pancreatitis

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INTRODUCTION

In a fashion similar to other solid organs, the pancreas has been linked with autoimmune disease in the form of "autoimmune pancreatitis". The concept was originally postulated by Sarles *et al*[1] over 40 years ago, when non-alcoholic pancreatitis was associated with hypergammaglobulinemia. However, only in the last 10 years has this clinical entity been firmly established in the list of diseases affecting the pancreas and thus appropriately gained significant attention. An efficient mimicker of pancreatic carcinoma on both clinical and radiologic grounds, its early and accurate diagnosis can have profound treatment and prognostic implications, distinguished by the rapid reversal of its characteristic lesions with corticosteroid therapy.

DEFINITION AND NOMENCLATURE

With mounting evidence suggesting an underlying autoimmune mechanism, the term autoimmune pancreatitis was originally introduced by Yoshida *et al*[2] in 1995. Characterized histologically by a predominant lymphoplasmacytic infiltrate and fibrosis that can lead to both endocrine and exocrine dysfunction, autoimmune pancreatitis likely accounts for a significant proportion of cases previously classified as idiopathic pancreatitis. Various disease descriptors have been previously proposed such as chronic sclerosing pancreatitis[3], lymphoplasmacytic sclerosing pancreatitis with cholangitis[4], sclerosing pancreatocholangitis[5], non-alcoholic duct-destructive chronic pancreatitis[6], chronic pancreatitis with irregular narrowing of the main pancreatic duct[7], and pseudotumorous pancreatitis[8]. Retrospectively, these various descriptors likely describe the entity that we now refer to as autoimmune pancreatitis, however focused on its specific radiologic or histologic findings. As will be discussed further, although autoimmune pancreatitis is now the preferred term, its clinical, biochemical, radiologic, and pathologic findings are heterogeneous.

DEMOGRAPHICS AND EPIDEMIOLOGY

Despite the increasing cases of autoimmune pancreatitis being reported around the world, its true prevalence and incidence have yet to be determined. Three case series have reported prevalence rates of 4% to 6% of all patients diagnosed with chronic pancreatitis[9,10]. The mean age of diagnosis is 55, this however can vary with cases presenting from 30 to 70 years of age[3,6,11,12]. A male predilection of 1.7:1-2:1 has been reported in three surgical series[12-14]. Considering its postulated autoimmune pathogenesis, it has been associated with Sjogren's syndrome[15,16], rheumatoid arthritis[14], primary sclerosing cholangitis[16,17], retroperitoneal fibrosis[18] and inflammatory bowel disease[19]. Although the prevalence of a concurrent autoimmune diagnosis has been reported in various series, they are likely underestimates as some diagnoses of autoimmune pancreatitis may precede the diagnosis of the concurrent autoimmune condition[9,10,20].

PATHOGENESIS

Although the precise etiology and pathogenesis of autoimmune pancreatitis remains unknown, mounting evidence supports an autoimmune cause. Similar to other autoimmune diseases, an association of the HLA haplotype DRB1*0405-DQB1*0401 with autoimmune pancreatitis in the Japanese population has been discovered[21]. Significant autoimmune markers of the disease include its association with other autoimmune diseases, a predominant lymphoplasmacytic infiltrate on histology, hypergammaglobulinemia, elevated IgG4 levels and the presence of autoantibodies. A number of autoantibodies including antinuclear antibody (ANA), antismooth muscle antibody (ASMA), rheumatoid factor, antilactoferrin antibody (ALF) and anticarbonic anhydrase Ⅱ antibody (CA-Ⅱ) have been frequently detected in patients with autoimmune pancreatitis[22]. Both CA-Ⅱ and ALF are found in the normal pancreas, with CA-Ⅱ located in duct cells and ALF being found in the acinar cells. However, they are also distributed in the cells of several other organs including the lactating breast, biliary ducts, distal renal tubules, and salivary, bronchial and gastric glands. Thus potentially explaining some of the extrapancreatic sequelae of autoimmune pancreatitis.

As in Sjogren's syndrome or primary sclerosing cholangitis, CD4+ T-cells inducing a Th1 type of immune response are predominantly involved in the development of autoimmune pancreatitis as effector cells over Th2 type CD4+ T-cells[22,23]. Further strengthening this discovery, an animal model of autoimmune pancreatitis, using neonatally thymectomized mice immunized with CA-Ⅱ or ALF found that CD4+ Th1 cells are mainly involved in the early development of murine autoimmune pancreatitis[24]. Pancreatic specimens from this mouse model revealed histologic features consistent with those seen in autoimmune pancreatitis in humans. Therefore, an autoimmune reaction against CA-Ⅱ or ALF *via* CD4+ Th1 T-cells has a role in the early development of autoimmune pancreatitis. However many questions remain regarding the underlying triggering event and precise mechanisms leading to autoimmune pancreatitis.

CLINICAL CHARACTERISTICS

The presenting symptoms are variable and most commonly include painless jaundice, weight loss and abdominal pain. Although common, abdominal pain tends to be mild and variable in duration, usually lasting weeks to months. Patients rarely present with acute attacks of pain, more typical of acute pancreatitis. Jaundice has been reported in up to 70%-80% of patients in some series and is usually due to an accompanying stricture in the distal common bile duct[25]. Considering the patient demographics and common presenting symptoms, it is clear why many patients are initially diagnosed with pancreatobiliary malignancies and prior to the recognition of autoimmune pancreatitis would undergo unnecessary invasive procedures.

In some series, up to 60% of patients with autoimmune pancreatitis have diabetes mellitus[26,27]. The precise pathogenesis is unclear however different theories have been proposed. The majority are felt to manifest as type 2 diabetics with a degree of impaired glucose tolerance, however a proportion have been found to have antibodies to islet cells and glutamic decarboxylase and thus can manifest as Type 1 diabetics. It has been reported that a proportion of patients with autoimmune pancreatitis associated diabetes mellitus improve following steroid therapy[28].

The presence of potential extrapancreatic target antigens in the lungs, breasts and kidneys can also lead to clinical manifestations. Breast, renal and pulmonary inflammatory masses have been detected in patients with autoimmune pancreatitis and found to be composed of a lymphoplasmacytic infiltrate containing numerous IgG4 positive plasma cells[29,30]. Cases of autoimmune pancreatitis associated with interstitial pneumonia and mild renal failure due to interstitial nephritis have also been reported[31-33].

DIAGNOSIS-DIAGNOSTIC IMAGING

Diagnostic imaging plays a critical role in the diagnosis of autoimmune pancreatitis. It is often the first investigative modality that raises the possibility of autoimmune pancreatitis, based on characteristic imaging features. As a result, both radiologists and non-radiologists need to be familiar with its unique features on ultrasound, computed tomography and endoscopic retrograde cholangio-pancreatography.

Ultrasound

Although abdominal ultrasonography is a commonly ordered initial radiologic investigation for painless jaundice, overlying bowel gas and obesity greatly limit its ability to accurately visualize the pancreas. Furthermore, ultrasound findings in patients with autoimmune pancreatitis are non-specific and include many features commonly seen in other types of acute and chronic pancreatitis. The role of contrast-enhanced ultrasonography is evolving and may prove to be an important modality in the differentiation of pancreatic lesions[34].

Computed tomography (CT)

In patients with diffuse pancreatic involvement, the findings on CT usually consist of a diffusely enlarged pancreas, commonly referred to as 'sausage-like' or 'bulky', which correlates with the pathologic finding of marked stromal edema on gross examination (Figure 1). Focal involvement typically appears as a mass, most commonly involving the head of the pancreas, with low attenuation or isoattenuation. Peri-pancreatic fat infiltration is uncommon. Pancreatic parenchymal calcification and intraductal stones, commonly seen in chronic pancreatitis, are also rarely observed in autoimmune pancreatitis. Mild peri-pancreatic lymphadenopathy and loss of parenchymal lobularity are commonly seen.

Another distinguishing feature of autoimmune pancreatitis is delayed enhancement of the pancreatic parenchyma. The pancreas tends to appear hypodense in comparison to the spleen on the arterial enhanced

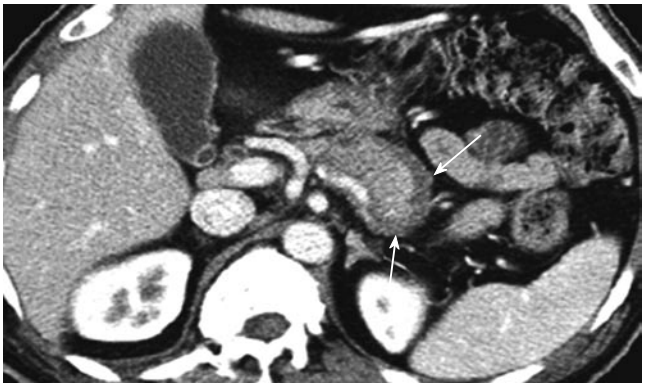

Figure 1 Contrast enhanced CT showing sausage-like swelling of the pancreatic tail along with a surrounding low attenuation rim (arrows).

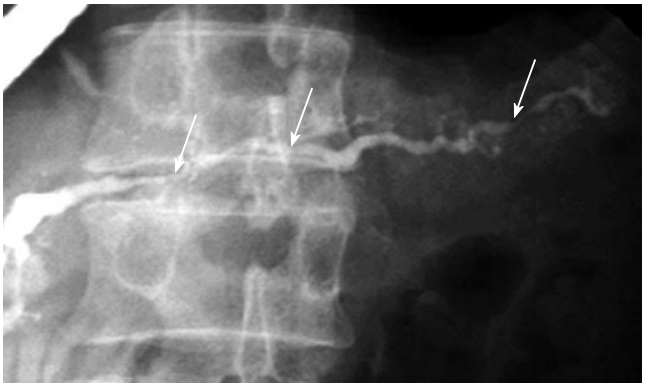

Figure 2 Direct pancreatography delineating a pancreatic duct with multiple segments of narrowing (arrows).

phase with increasing attenuation in the delayed phase images. It has been proposed that delayed enhancement is a reflection of the heterogeneous lymphoplasmacytic infiltrate and fibrosis observed on histologic examination of the pancreas[35]. Finally, a capsule-like low density rim can also be seen surrounding the pancreas on both arterial and delayed images. This may represent clear demarcation of the inflammatory process in autoimmune pancreatitis from the surrounding peripancreatic fat, due to the absence of peripancreatic fat infiltration.

Magnetic resonance cholangio-pancreatography (MRCP)

The role of MRCP continues to evolve, especially as a non-invasive modality in assessing pancreatobiliary ductal anatomy. Its role however in autoimmune pancreatitis remains limited, primarily because the lack of exogenous contrast and lower resolution compared to ERCP, greatly limit its ability to detect the characteristic pancreatic duct changes.

Endoscopic retrograde cholangio-pancreatography (ERCP)

The hallmark findings on direct pancreatography are focal, segmental, or diffuse narrowing of the main pancreatic duct (Figure 2)[36]. The pattern and degree of ductal narrowing is a direct result of ductal compression due to the heterogenous lymphoplasmacytic infiltrate and fibrosis affecting the gland. Although uncommon, the focal variant of autoimmune pancreatitis represents a diagnostic challenge because its findings are quite similar to those expected in pancreatic cancer. Usually associated with a mass in the head of the pancreas on CT, the focal type of autoimmune pancreatitis results in localized stenosis of the main pancreatic duct and upstream dilatation.

In the 'segmental' variant, multiple strictures affecting different sections of the pancreatic dust are visualized with intervening duct that appears non-dilated. In the 'diffuse' form, the entire pancreatic duct is narrowed. Rather than representing distinct variants of autoimmune pancreatitis, some reports have documented progression of the segmental form to a more diffuse appearance on ERCP without therapy[12]. Thus suggesting that the different patterns of ductal narrowing are primarily a result of the timing of ERCP and represent the spectrum of the disease rather than distinct entities[20,36].

Other potential ERCP findings in autoimmune pancreatitis include narrowing of the intrapancreatic portion of the common bile duct and irregular narrowing of extrahepatic bile ducts. Although uncommon, dilatation of the intrahepatic bile ducts has also been reported[36].

Endoscopic ultrasonography (EUS)

Due to its ability to accurately visualize the pancreas and perform fine needle aspiration, EUS is a crucial modality in the diagnosis of autoimmune pancreatitis. The absence of exogenous contrast makes it an important diagnostic test, even prior to CT, especially in women of child-bearing age. The findings seen in autoimmune pancreatitis closely resemble those found on CT, including a hypoechoic gland with focal or diffuse parenchymal swelling. Above all, the ability to perform tissue acquisition either by fine needle aspiration or core biopsy further strengthens the role of EUS in the diagnosis of this elusive entity.

DIAGNOSIS-PATHOLOGY

The hallmark histologic finding of autoimmune pancreatitis includes a dense lymphoplasmacytic infiltrate and fibrosis (Figure 3). The infiltrate is often heterogeneous in its distribution throughout the gland and its degree of cellularity. Although consisting predominantly of lymphocytes, the cellular infiltrate also contains neutrophils and eosinophils. Studies using immunohistochemistry have further defined that the lymphocyte population consists predominantly of CD4+ T lymphocytes with a smaller but detectable population of CD8+T cells and B cells[24].

Another important histologic finding is the presence of periductal inflammation, usually involving medium-sized and large interlobular ducts (Figure 4)[37]. Sometimes referred to as a 'collar' of inflammation, it consists predominantly of plasma cells and lymphocytes, sometimes forming germinal centers. The infiltrate is primarily subepithelial, with the epithelium only rarely being infiltrated by lymphocytes. It completely encompasses the duct leading to luminal narrowing by way of epithelial infolding and gives the lumen a star-like appearance[24]. Periphlebitis and perineural inflammation are also often

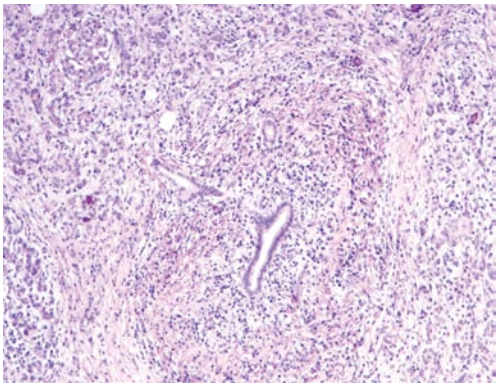

Figure 3 Diffuse pancreatic lymphoplasmacytic infiltrate with early fibrosis and atrophy.

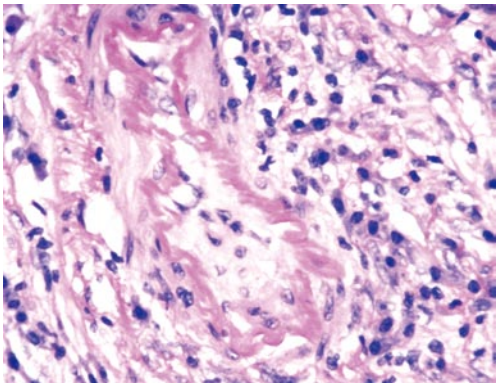

Figure 5 Medium sized artery with infiltration of the vessel wall by lymphocytes and plasma cells.

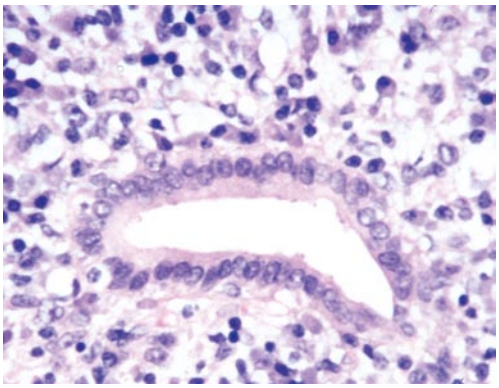

Figure 4 Small pancreatic duct surrounded by a cuff of lymphocytes and plasma cells that is extending into the atrophic pancreatic parenchyma.

Table 1 Diagnostic criteria for autoimmune pancreatitis proposed by the Japanese Pancreas Society

A Imaging criterion: Diffuse narrowing of the main pancreatic duct with an irregular wall (more than 1/3 the length of the entire pancreatic duct) and enlargement of the pancreas
B Laboratory criterion: Abnormally elevated levels of serum gamma-globulin and/or IgG, or the presence of autoantibodies
C Histopathologic criterion: Marked lymphoplasmacytic infiltration and dense fibrosis
For the diagnosis, criterion A must be present together with criterion B and/or C

observed (Figure 5)[12,30].

Finally, an important histologic observation in the pathologic diagnosis of autoimmune pancreatitis is the absence of ductal dilatation, calcifications and proteinaceous plugs, commonly found in chronic pancreatitis.

DIAGNOSTIC CRITERIA

Considering the difficulty in accurately differentiating autoimmune pancreatitis from other forms of chronic pancreatitis and pancreatic carcinoma, a number of diagnostic criteria have been proposed. The earliest was proposed by the Japanese Pancreas Society and is outlined in Table 1[38]. It is based on three criteria encompassing imaging, laboratory and histopathologic findings. The mandatory imaging findings include diffuse narrowing of the pancreatic duct involving at least one third of the entire length along with pancreatic enlargement. One of the two 'minor' laboratory or pathologic criteria also needs to be present for the diagnosis. Aparisi *et al*[39] proposed a different scoring system based on clinical presentation, laboratory parameters and morphologic findings. More recently, investigators have specifically included an elevation in the IgG4 level and response to steroids as supporting criteria for the diagnosis of autoimmune pancreatitis[11,40].

Kim *et al*[11] retrospectively applied the original diagnostic criteria of the Japanese Pancreas Society to a series of 28 patients diagnosed with autoimmune pancreatitis who had responded to steroid therapy. The original diagnosis required the presence of diffuse enlargement of the pancreas and diffuse or segmental irregular narrowing of the main pancreatic duct. Supporting criteria included elevated levels of IgG and/or IgG4, the presence of autoantibodies and histopathologic findings. Despite the absence of laboratory abnormalities or histopathologic findings in some patients, steroid therapy was initiated if the imaging criteria were met. The authors concluded that had they strictly applied the diagnostic criteria of the Japanese Pancreas Society, 9 of 28 patients would have not been diagnosed with autoimmune pancreatitis and appropriately treated. Seven of the 9 patients would have been missed because the extent of ductal narrowing was less than one third of the entire length of the main pancreatic duct. Another two patients had normal IgG levels, absence of autoantibodies and non-diagnostic pancreatic histopathology. As a result, the authors revised the diagnostic criteria by including the response to steroids and association with other autoimmune diseases as supporting criteria. They also abolished the need for more than one third of the pancreatic duct to be affected.

Although none of the proposed diagnostic criteria have been validated, they underscore the highly characteristic

imaging findings in autoimmune pancreatitis. Finally, the histopathologic findings which have previously been felt to represent the gold standard for the diagnosis can be absent on specimens due to the patchy distribution of the disease and have also been seen in patients with alcohol induced chronic pancreatitis[30].

TREATMENT AND PROGNOSIS

Corticosteroids are the first line therapy in the treatment of autoimmune pancreatitis, often providing dramatic and rapid results. The role of other immunosuppressive agents in this patient population remains largely undefined.

Prednisone is usually initiated at a dose of 0.4-0.6 mg/kg per day for a period of months. Although a detailed steroid schedule has not yet been fully defined, most patients are usually treated for a period of 2-3 mo, with a tapering schedule of 5 mg every 1-2 wk.

From a laboratory perspective, hypergammaglobulinemia and elevated IgG4 levels can resolve and previously identified autoantibodies can become undetectable[41]. Marked changes are also observed in the pretreatment findings of CT and ERCP[20]. The diffuse enlargement of the gland, characteristic pattern of enhancement and capsule-like low density rim seen on CT revert to normal. ERCP findings of focal, segmental or diffuse pancreatic duct narrowing also disappear. Narrowing of the distal common bile duct normalizes. Since almost all patients undergo ERCP prior to the initiation of steroid therapy, the finding of distal common bile duct narrowing is often treated with stent placement. The rapid reversal of biliary narrowing with steroid therapy usually allows for stent removal within 1-2 mo.

These radiologic changes closely parallel the clinical improvement that patients experience, specifically regarding the most common presenting symptoms of abdominal pain or painless jaundice. They are often observed within 2-4 wk of corticosteroid initiation and serve to further confirm the diagnosis of autoimmune pancreatitis.

In terms of prognosis, although the majority of patients achieve a sustained clinical remission with a tapering course of corticosteroid therapy, a subset may go on to require chronic maintenance dosing of 5-10 mg/d. The long term prognosis of autoimmune pancreatitis remains as yet undefined considering its recent discovery. Two different cohorts involving 23 patients followed for a mean of 56 mo and 17 patients followed for 16 mo, each had 1 patient relapse[9,35]. In both cases the patient was successfully treated with a second course of corticosteroids and maintained on low dose steroid therapy.

CONCLUSION

In the last 40 years autoimmune pancreatitis has gone from a proposed concept to a well-recognized clinical entity. Despite the fact that our understanding regarding its pathogenesis, presentation, diagnosis and treatment have evolved, many questions remain unanswered. Its relationship to other autoimmune diseases, precise pathogenesis, accurate diagnosis and long-term prognosis require further clarification.

Above all, as an efficient mimicker of pancreatic carcinoma, the accurate and timely diagnosis of autoimmune pancreatitis can have drastic consequences on therapy and prognosis. Thereby underlining the critical importance of its awareness among internists, gastroenterologists, radiologists, pathologists and surgeons.

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TOPIC HIGHLIGHT

Michael F Byrne, MD, *Series Editor*

Sphincter of Oddi dysfunction and pancreatitis

MT McLoughlin, RMS Mitchell

MT McLoughlin, RMS Mitchell, Department of Gastroenterology, Belfast City Hospital, Northern Ireland
Correspondence to: Dr. RMS Mitchell, Consultant Gastroenterologist, Belfast City Hospital Trust, Lisburn Road, Belfast, BT9 7AB, Northern Ireland. michael.mitchell@bch.n-i.nhs.uk
Telephone: +44-28-90263573 Fax: +44-28-90263973
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Abstract

Sphincter of Oddi dysfunction (SOD) is a term used to describe a group of heterogenous pain syndromes caused by abnormalities in sphincter contractility. Biliary and pancreatic SOD are each sub-classified as type Ⅰ, Ⅱ or Ⅲ, according to the Milwaukee classification. SOD appears to carry an increased risk of acute pancreatitis as well as rates of post ERCP pancreatitis of over 30%. Various mechanisms have been postulated but the exact role of SOD in the pathophysiology of acute pancreatitis is unknown. There is also an association between SOD and chronic pancreatitis but it is still unclear if this is a cause or effect relationship. Management of SOD is aimed at sphincter ablation, usually by endoscopic sphincterotomy (ES). Patients with type Ⅰ SOD will benefit from ES in 55%-95% of cases. Sphincter of Oddi manometry is not necessary before ES in type Ⅰ SOD. For patients with types Ⅱ and Ⅲ the benefit of ES is lower. These patients should be more thoroughly evaluated before performing ES. Some researchers have found that manometry and ablation of both the biliary and pancreatic sphincters is required to adequately assess and treat SOD. In pancreatic SOD up to 88% of patients will benefit from sphincterotomy. Therefore, there have been calls from some quarters for the current classification system to be scrapped in favour of an overall system encompassing both biliary and pancreatic types. Future work should be aimed at understanding the mechanisms underlying the relationship between SOD and pancreatitis and identifying patient factors that will help predict benefit from endoscopic therapy.

Key words: Sphincter of Oddi dysfunction; Pancreatitis, Post-ERCP pancreatitis; Sphincter of Oddi manometry; Endoscopic sphincterotomy

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INTRODUCTION

Sphincter of Oddi dysfunction (SOD) is the term used to describe a heterogenous group of clinical pain syndromes caused by abnormalities in sphincter contractility. The sphincter of Oddi (SO), a fibromuscular sheath encircling the distal common bile duct (CBD), pancreatic duct (PD) and common channel, controls the flow of bile and pancreatic secretions into the duodenum and prevents reflux of duodenal contents into the pancreaticobiliary system.

SOD describes SO dysmotility or stenosis leading to reduced transphincteric flow of bile or pancreatic juice[1]. SO stenosis is a structural abnormality where there is a physical alteration of the sphincter due to inflammation and fibrosis. SO dyskinesia results in a hypo- or hypertonic sphincter with altered motility causing an intermittent functional blockade of the sphincter[2]. As it is often difficult to distinguish SO stenosis from dyskinesia, the term Sphincter of Oddi dysfunction is used to cover both conditions.

Because of the anatomical position of the SO patients with SOD typically present with recurrent biliary or pancreatic type pain. The Rome Ⅱ diagnostic criteria for biliary pain are episodes of severe steady pain in the epigastrium and right upper quadrant, associated with all of the following: (1) Symptom episodes lasting at least 30 min with pain free episodes in between; (2) At least one attack of pain in the last 12 mo; (3) Pain that is steady and interrupts daily activities or requires consultation with a doctor; (4) No evidence of structural abnormalities to explain the symptoms.

Pancreatic pain is described as post-prandial, episodic, prolonged pain in the upper abdomen and/or back[3]. It is often presumed in the setting of acute recurrent pancreatitis in the absence of biliary stone disease or anatomical abnormalities. The true prevalence of SOD is not known but ongoing biliary type pain occurs in 10%-20% of patients who have had a cholecystectomy[4]. Sphincter ablation, usually by endoscopic sphincterotomy, is at the forefront in the management of SOD and one of the challenges of this condition is to identify which patients will benefit from it.

CLASSIFICATION OF SOD

The Milwaukee Classification, proposed by Hogan and Geenen[5,6], sub-classifies biliary and pancreatic SOD into three types on the basis of symptoms, laboratory tests and radiological imaging (Table 1). Abnormally high basal sphincter pressure identified during sphincter of Oddi manometry (SOM) confirms the presumed diagnosis. As biliary drainage time is difficult and somewhat impractical to measure and may increase the risk of an ERCP it is rarely performed in clinical practice. In any case, there may be little or no correlation between basal sphincter of Oddi pressures and drainage times[7]. Therefore, a contemporary modified version of the Milwaukee classification, which does not include duct drainage times, is generally used in practice[3]. Sub classification of SOD into types Ⅰ, Ⅱ and Ⅲ helps predict the underlying pathology and the likelihood of symptom relief after treatment. Type Ⅰ disease is thought to result from a fixed stenosis caused by chronic inflammation and fibrosis and has the highest response rate to therapy. An episodic dysmotility is the presumed underlying abnormality in the other types and often does not respond as well to treatment[8,9].

There are some potential problems with the Milwaukee classification. For example, the description of typical biliary or pancreatic pain may be interpreted differently between individuals and this may lead to inappropriate referral for SOM, particularly for patients with presumptive type Ⅲ SOD. Also, according to the Milwaukee criteria, LFTs should normalize between attacks but patients are often labeled with type Ⅱ SOD on the basis of pain and abnormal LFTs which do not normalize[10]. CBD diameter of at least 12 mm is one of the criteria in the diagnosis of SOD. Most patients being investigated for SOD have had their gallbladder removed and in the past it was accepted that it was normal for a post-cholecystectomy CBD to be 2-3 mm dilated. However, in a cohort of 59 patients, Majeed *et al*[11] found no difference between pre- and post-cholecystectomy CBD diameter. As the upper limit of normal for CBD diameter is 7 mm, a cut off of 12 mm potentially leaves a large number of patients misdiagnosed. Also, variations in basal pressure and response to sphincterotomy between the biliary and pancreatic portions of the SO have led to calls for this dual classification system to be scrapped in favor of a single, overall system.

Table 1 Milwaukee classification of sphincter of Oddi dysfunction

1 Biliary type:
Type Ⅰ:
Typical biliary type pain
Liver enzymes (AST, ALT or ALP) > 2 times normal limit documented on at least 2 occasions during episodes of pain
Dilated CBD > 12 mm in diameter
Prolonged biliary drainage time (> 45 min)
Type Ⅱ:
Biliary type pain and
One or two of the above criteria
Type Ⅲ:
Biliary type pain only
2 Pancreatic type SOD
Type Ⅰ:
Pancreatic type pain
Amylase and/or lipase > 2 times upper normal limit on at least 2 occasions during episodes of pain
Dilated pancreatic duct (head > 6 mm, body > 5 mm)
Prolonged pancreatic drainage time (> 9 min)
Type Ⅱ:
Pancreatic type pain, and
One or two of the above criteria
Type Ⅲ:
Pancreatic type pain only

SPHINCTER OF ODDI MANOMETRY

SOM remains the gold standard for the diagnosis of SOD. It is usually combined with a diagnostic ERCP examination and involves cannulating the ampulla with the manometry catheter. A triple lumen catheter allows continuous aspiration of PD fluid that may reduce the risk of post- procedural pancreatitis[12]. To determine which duct has been cannulated a small amount of contrast is injected or some fluid aspirated to determine its color. A catheter "pull-through" of the sphincter is performed to assess the pressure profile and to localize the point of peak basal pressure. Normal basal sphincter pressure is approximately 15 mmHg but ranges from 3 to 35 mmHg. It is generally accepted that a basal pressure greater than 40 mmHg (based on a threshold of 3 standard deviations above the median) is abnormal[13]. In patients with SO stenosis this recording is reproducible and does not respond to muscle relaxants[1]. In contrast, SO dyskinesia is characterized by a response to smooth muscle relaxants[5], an excess of retrograde contractions (> 50%), tachyoddia (rapid contraction frequency > 7/min) and a paradoxical contraction response of the SO following an intravenous dose of CCK[1,14].

In type I SOD SOM will be abnormal in 75%-95%[15]. However, the frequency of abnormal biliary manometry varies from 28% to 60% for type Ⅱ patients and from 7 to 55% in type Ⅲ patients[16]. Various factors may explain the differences in frequencies of SOD in published reports. For example, selection of patients with a typical biliary or pancreatic type pain rather than a non-specific pain will increase the yield of basal pressure abnormality. SOM measures a "snap shot" of sphincter pressure during the study period that may not always be reproducible. A study of 12 patients with previously normal SOM showed evidence of elevated SO pressures in 5 (42%) when re-tested after a median of 337 d[17]. Also, the pressure in the pancreatic and biliary portions of the SO can vary so assessment of only one sphincter component, rather than both, will reduce the frequency of SOD detection. Current data suggests a discordance rate of between 35% and 65%[16,18-22]. Therefore, both portions of the SO should be measured separately for a full assessment. This necessitates classifying each patient with respect to the pancreatic and biliary components of the SO and is one of the reasons some experts have called for a single overall classification system. When both sides of the sphincter are evaluated there is little difference between them in predicting

abnormal basal pressure[16].

Because SOM is technically difficult, invasive, has a variable diagnostic yield and has recognized complications, other indirect methods of evaluating SO function have been developed. These include the Morphine-Prostigmin provocative test (Nardi test; now obsolete), the ultrasound- or MRCP-secretin test, and quantitative hepatobiliary scintigraphy. However, current data suggests that non-invasive tests have a relatively low specificity and sensitivity[15], although there is some evidence that secretin stimulated MRCP may be useful in selecting patients with suspected type Ⅱ SOD who are most likely to benefit from sphincterotomy [23]. Therefore, despite the risk, and assuming careful patient selection, SOM remains the diagnostic tool of choice for most clinicians.

PANCREATITIS POST SOM

Acute pancreatitis is the main complication of SOM. Increased intraductal pressure, overfilling of the ductal system, difficult and repeated cannulation of the PD causing spasm and trauma have all been postulated as etiological factors, possibly by affecting pancreatic duct drainage[24]. This hypothesis is indirectly supported by the observation that PD stenting after biliary sphincterotomy[24] and needle knife sphincterotomy over a PD stent[25] have been found to reduce the incidence of pancreatitis in patients with SOD.

The rate of post-SOM pancreatitis in patients suspected of having SOD has been found to be as high as 31%[27-30]. Sherman *et al*[27] found a much lower rate of pancreatitis when an aspirating catheter was used (1 of 33 patients; 4%) compared with an infusion catheter (8 of 34 patients; 31%). Walters *et al*[31], however, found no difference in the incidence of pancreatitis when comparing the two types of manometry catheter (8% *vs* 13%). In a case series of 146 patients (207 SOM measurements), Rolny *et al*[28] reported a 6% incidence of pancreatitis when using the standard catheter. In addition, acute pancreatitis developed in 10 of 95 (11%) patients who had undergone pancreatic manometry alone, compared with 1 of 93 (1%) who had biliary manometry alone. Recommended methods of reducing the rate of pancreatitis from SOM include evaluating biliary SO alone in patients with suspected biliary disease[32], limiting SO perfusion to 1-2 min[33] and careful patient selection. For example, Scicchitano *et al*[29] found a significantly higher rate of pancreatitis when the indication for SOM was idiopathic acute recurrent pancreatitis (IARP) compared to unexplained abdominal pain (29% *vs* 6%). The incidence of pancreatitis was 50% in the patients with IARP and high SO basal pressure. Temporary prophylactic pancreatic duct stenting has been shown to reduce the incidence of pancreatitis in a variety of patient groups, including those undergoing SOM[25,26,34,35].

A retrospective review of 100 patients who had undergone SOM found an overall incidence of pancreatitis of 17%[30]. The incidence was significantly higher in patients who had undergone SOM and ERCP, compared to those who had only undergone SOM (26.1% *vs* 9.3%). Multiple regression analysis showed that sphincterotomy added no additional risk beyond that associated with ERCP. These results imply that other factors during ERCP, and not the manometry itself, predispose to pancreatitis. The authors recommended that ERCP should be performed at another session, possibly 24 h after SOM.

Results from other studies suggest that the risks of pancreatitis are intrinsic to the patient group undergoing the procedure and the therapy provided, rather than the SOM itself. Freeman *et al*[36] recorded complication rates for sphincterotomy in patients with suspected SOD and those in whom it was already confirmed. The complication rate was 21% for patients who underwent SOM and 25% when sphincterotomy was not preceded by SOM. Another study compared the pancreatitis rate from ERCP between patients with suspected SOD, some of whom also underwent SOM, and a control group of patients with biliary stones[37]. 27% of patients with suspected SOM developed post-procedural pancreatitis, compared with 3.2% of the control group ($P < 0.001$). However, there was no significant difference in the rate of acute pancreatitis in the first group between those who had SOM and those who did not (OR 0.72: 95% CI 0.08-9.2). Similarly, in a large trial of over 1000 patients who underwent ERCP with or without SOM, Cheng *et al*[38] found that SOM was not a risk factor for post-ERCP pancreatitis.

The variability in complication rates between studies is probably multifactorial and related to the timing and duration of the procedure, the number of passes with the manometry catheter and technique and skill of the operator. However, it is probable that, in skilled hands, SOM does not significantly increase the risks of post-ERCP pancreatitis and remains a useful tool in the diagnosis of SOD, particularly for types Ⅱ and Ⅲ.

SOD AND ACUTE PANCREATITIS

SOD may contribute to the risk of acute pancreatitis by causing abnormal biliary or pancreatic juice flow. In the Australian Bush opossum, which has a similar biliary and pancreatic anatomy to humans, the combination of pancreatic duct ligation and stimulation of pancreatic exocrine secretion with cholecystokinin/secretin uniformly causes acute pancreatitis[39]. In another group, reduced transphincteric flow was achieved by applying topical carbachol to the SO, causing PD pressures comparable with those opossums in which the PD was ligated. However, acute pancreatitis only occurred when carbachol application was combined with pancreatic secretory stimulation. Decompression of the PD negated the effects. Therefore, the combination of PD obstruction with increased exocrine secretion was needed to produce acute pancreatitis. Although it is a recognized complication of SOD, this study demonstrated that SOD might be a causative factor in the production of acute pancreatitis.

Kruszyna *et al*[40] carried out ERCP with pre- and post- sphincterotomy SOM in a group of 30 patients with mild acute biliary pancreatitis and compared results with a control group of 30 patients with no evidence of CBD stones or pancreatitis. The patients with pancreatitis had a significantly elevated CBD pressure, SO basal pressure and wave amplitude compared to controls.

There was a significant reduction in all parameters after sphincterotomy. They concluded that SO dysfunction, either primary or secondary to spasm caused by a gallstone migrating through the ampulla, may have a role in acute biliary pancreatitis.

Although there is very little direct evidence supporting the role of the SO in causing pancreatitis in humans, there is plenty of circumstantial evidence. Fazel *et al*[41] measured intrapancreatic ductal pressure blindly in 263 patients presenting with either recurrent abdominal pain, acute recurrent pancreatitis or chronic pancreatitis. Complete SOM was then performed and patients with SOD were found to have a significantly higher ductal pressure compared to those with normal SO motility. This difference was seen across all three groups ($P < 0.01$) and patients with acute and chronic pancreatitis did not have a significant elevation in intraductal pressure compared to individuals with abdominal pain only. The authors concluded that SOD leads to an increase in intrapancreatic ductal pressure but this rise in pressure is not the sole cause of pancreatitis.

Warshaw *et al*[42] showed that infusion of secretin caused PD dilatation of > 1 mm in 83% of patients with SO stenosis and 72% with accessory papilla stenosis, compared with controls. This dilatation response was abolished after surgical sphincteroplasty. A positive secretin test was associated with a good surgical outcome in 90% of cases. It has been shown that in patients undergoing surgery for idiopathic acute recurrent pancreatitis (IARP) the SO narrows at the opening of the PD, suggesting that this narrowing may play a role in its development[43].

An abnormality of SO function has also been implicated in the pathogenesis of acute pancreatitis attributed to other causes. An organophosphate insecticide is a recognized cause of acute pancreatitis in humans. It acts by irreversibly inhibiting cholinesterase resulting in delayed breakdown of synaptic acetylcholine[44], and has been shown to cause pancreatitis in animals[45], probably due to the combination of obstruction at the level of the SO and cholinergic stimulation of pancreatic secretions. Scorpion venom causes acetylcholine release, stimulating the pancreas and SO, and causes pancreatitis in a similar way to organophosphate poisoning[46].

Other rare causes of acute pancreatitis including hypercalcemia and hyperlipidemia may involve abnormalities of SO function. High extra-cellular calcium stimulates smooth muscle and stimulates pancreatic secretion in animal models and it is thought that abnormal calcium regulation of the SO may be an underlying factor in the pathophysiology[47]. A study of hypercholesterolemic rabbits showed a failure of SO relaxation again indirectly suggesting that SO dysfunction may contribute to the risk of pancreatitis[48]. Therefore, although its exact role is not known, the evidence, taken together, suggests that the SO at some level is an important factor in the development of acute pancreatitis, including pancreatitis that may be attributed to another aetiology.

SOD IN RECURRENT ACUTE PANCREATITIS

Clinical evaluation, blood testing and imaging will yield a cause of acute recurrent pancreatitis in 70%-90% of cases. In the remaining "idiopathic" acute recurrent pancreatitis (IARP) cases more extensive evaluation may be required, including assessment for SOD. Abnormal SOM in IARP ranges from 15%-72% with a mean of 30.5% (Table 2)[16,49-54]. The high incidence of abnormal SOM in IARP reflects the fact that a substantial proportion of these patients are likely to have SOD .

Table 2 Frequency of abnormal sphincter of Oddi manometry in idiopathic acute recurrent pancreatitis

Author	Year	Patient number, *n*	Abnormal SOM	Frequency (%)
Gregg *et al*[49]	1984	125	28	22
Toouli *et al*[50]	1985	28	14	50
Venu *et al*[51]	1989	116	17	15
Sherman *et al*[52]	1992	49	15	31
Eversman *et al*[16]	1999	47	34	72
Coyle *et al*[53]	2002	90	28	31
Kaw *et al*[54]	2002	126	41	33
Total		581	177	30.5

With the exception of a study by Eversman *et al*[16], the published studies measured sphincter pressure in only one duct, i.e., either pancreatic OR biliary, although in some cases it is not clear which duct was actually measured. Eversman *et al*, however, performed SOM of the biliary and pancreatic ducts in 593 patients, of whom 360 had intact sphincters. Of the 47 patients with idiopathic acute pancreatitis, 12 had increased pressure in the pancreatic portion of the SO, 3 had increased pressure in the biliary portion and 19 had it in both. The measurement of sphincter pressure in both ducts accounts for the much higher frequency of SOD in IARP that was found in this study. Choudari *et al*[55] also reported a higher frequency of basal sphincter abnormality of at least one duct in patients with chronic pancreatitis.

Of the 360 patients measured in the study by Eversman *et al*[16], 68 (18.9%) had abnormal pancreatic sphincter basal pressure alone, 41 (11.4%) had abnormal biliary basal sphincter pressure alone and in 113 (31.4%) the basal pressure was abnormal for both sphincters. Therefore, 219 (60.1%) of the patients had sphincter dysfunction. The authors concluded that assessment of both the pancreatic and biliary portions of the SO is necessary to accurately detect SOD. The frequency of SOD did not differ whether typed by biliary or pancreatic criteria (65% type Ⅱ and 59% type Ⅲ). As there was so little difference in the frequency of SOD according to the modified Geenen-Hogan criteria, the authors argued for an overall classification for SOD encompassing biliary and pancreatic types.

Guelrud *et al*[56] retrospectively reviewed ERCP studies from 64 children (> 1 year old) and adolescents with recurrent pancreatitis. SOM and sphincterotomy were performed in 9 patients, all of whom had SOD. Seven of these patients had a choledochal cyst and 2 had anomalous pancreaticobiliary union (APBU). After a mean follow up of 26.4 mo (range 18-38), 8 of these patients were symptom free and one had occasional pain but no further episodes of pancreatitis. They concluded that recurrent

pancreatitis and ABPU are associated with SOD in children and adolescents and that sphincterotomy was beneficial to these patients.

SOD AND CHRONIC PANCREATITIS

Early studies investigating the association of SOD and chronic pancreatitis were inconclusive. Some studies showed no difference in pancreatic sphincter pressures between patients with chronic pancreatitis and controls[57-61]. However, these studies involved patients with chronic pancreatitis due to alcohol and in two of the studies the controls were patients with unexplained abdominal pain[58] or suspected biliary dyskinesia[60]. Also, although one of these studies found no significant difference between SO basal pressure in patients with chronic pancreatitis and controls, the pancreatic duct pressure was significantly higher in the early stages of chronic pancreatitis than normal subjects[57]. Other trials have shown a correlation between elevated pancreatic sphincter pressures and chronic pancreatitis[19,62-64]. Many of these also used patients with chronic pancreatitis secondary to alcohol. However, in the only one of these studies that excluded alcoholic patients, basal pancreatic sphincter pressures were significantly higher in the early stages of chronic pancreatitis than controls[62]. Laugier[64] performed manometry of the SOD and main pancreatic duct before and after intravenous injection of secretin in chronic pancreatitis patients and controls. Secretin transiently increased pancreatic duct pressure in controls, but chronic pancreatitis patients had a persistently elevated pancreatic duct pressure and a manometric pattern of SOD. The secretin-induced elevation in ductal pressure was greater and more sustained in patients with chronic pancreatitis, particularly of recent onset (less than 4 years).

It has been shown that local installation of alcohol on the SO results in elevated SO pressures, suggesting a role in the pathogenesis of alcoholic pancreatitis[65]. Tarnasky *et al*[66] looked for evidence of chronic pancreatitis in patients undergoing manometry for investigation of unexplained upper abdominal pain (n = 104). Pancreatic ductography, EUS and pancreatic fluid bicarbonate concentration measurements were carried out. Patients with SOD were 4 times more likely to have evidence of chronic pancreatitis than those with normal sphincter pressure (P = 0.01). Of 68 patients with SOD, 20 (29%) had structural evidence of chronic pancreatitis and 20 of 23 patients (87%) with chronic pancreatitis had SOD. The authors concluded that SOD is associated with structural evidence of chronic pancreatitis in patients with unexplained pancreaticobiliary pain. Patients with chronic pancreatitis and SOD were significantly older than those with SOD but no chronic pancreatitis. This raises the possibility that SOD precedes the development of pancreatitis.

The available evidence certainly suggests a link between SOD and chronic pancreatitis. However, it is still not clear if this is a cause or effect relationship, i.e., does the generalized scarring associated with chronic pancreatitis also involve the sphincter or does the hypertensive sphincter cause elevated pressure and, hence, morphological changes? Further work is required to clarify this issue.

SPHINCTEROTOMY FOR SOD

Biliary type SOD

Management of SOD has traditionally been aimed at sphincter ablation by endoscopic sphincterotomy. Most data on sphincterotomy relates to biliary sphincter ablation alone and clinical improvement has been reported to occur in 55%-95% of patients[15] with the grade of SOD having a significant effect on outcome. Outcomes are generally measured using pain scores or quality of life measures[2], although a lack of standardization in characterizing the patients and assessing response make comparisons between trials problematic.

There are no randomized or controlled trials of therapy for type I SOD and the available evidence is derived from small retrospective trials. Rolny *et al*[67] carried out ERCP and SOM on 17 post-cholecystectomy patients with suspected type I SOD. All patients had a dilated CBD at ERCP and delayed contrast drainage and 11 had elevated SO pressure. Sphincterotomy resulted in symptom relief in all patients after a mean follow up of 28 mo. It was concluded that, in symptomatic post-cholecystectomy patients, the triad of abnormal LFTs, dilated CBD and delayed contrast drainage was sufficient to make a diagnosis of definitive SO abnormality and, as these patients invariably benefit from sphincterotomy, SOM was unnecessary.

Other studies have reported the effect of sphincterotomy for both type I and type Ⅱ patients. Thatcher *et al*[68] retrospectively reviewed 46 patients (31 with type I and 15 with type Ⅱ) who had undergone sphincterotomy for SOD. In the patients with type I SOD 87% had improved pain scores at 3 mo and 77% after a mean follow up of 12.5 mo. When evaluated along with the patients with type Ⅱ SOD, patients with a dilated bile duct and delayed contrast drainage at ERCP had a better response to therapy (P = 0.01) and reduced complication rate (P = 0.03) compared to those with normal ducts at ERCP. 29 patients underwent SOM but a favorable treatment outcome did not correlate with manometric assessment, particularly in patients with abnormal ducts. Therefore, patients suspected of having type I SOD benefited from sphincterotomy, irrespective of SOM results.

Lin *et al*[69] performed sphincterotomy on 24 patients based on clinical findings of post-cholecystectomy pain, biochemical abnormalities and/or dilated bile ducts. Enzyme abnormalities were a significant predictor of response to therapy (P = 0.018) whereas duct dilatation was not (P = 1.0).

These small studies suggest that endoscopic sphincterotomy without SOM is effective in suspected type I biliary SOD. However, patients with presumptive type Ⅱ SOD have, by definition, less concrete evidence for obstruction at the level of the sphincter so more extensive evaluation is necessary to predict those who would benefit from sphincterotomy.

Three randomized trials of endoscopic therapy for types Ⅱ and Ⅲ SOD have been reported. In one of these

47 patients with presumed type Ⅱ SOD were randomly assigned to endoscopic sphincterotomy (n = 23) or a sham procedure (n = 24) in a prospective double-blind study[6]. All patients had biliary type pain, clinical characteristics in keeping with biliary obstruction and had a previous cholecystectomy. Eleven patients in the treatment group had manometric evidence of elevated sphincter pressure and 10/11 described improved pain scores at 1 year. In contrast, only 3 out of 12 patients in the control group who had elevated pressure had an improved pain score over the same time period. Pain scores were unchanged in patients with normal sphincter pressures, irrespective of treatment. After one year sphincterotomy was performed in 12 symptomatic patients who had initially undergone the sham procedure, 7/12 with elevated sphincter pressure and 5/12 with normal pressure. A total of 40 patients were followed for 4 years and after that time 17 of the 18 patients (95%) with SOD verified by manometry had benefited from sphincterotomy. However, only 30%-40% of patients with an elevated sphincter pressure treated with sham sphincterotomy or with a normal pressure treated by sphincterotomy or sham benefited from therapy. The authors concluded that SOM predicted outcome from sphincterotomy and that sphincterotomy offers long-term pain relief in patients with verified SOD.

An Australian study of SOM in 81 post-cholecystectomy patients with biliary-type pain compared outcomes among a mixed group of patients with types Ⅰ (n = 9), Ⅰ-Ⅱ (n = 27), Ⅱ (n = 27) and Ⅲ (n = 18)[70]. The manometric records were categorized as SO stenosis, SO dyskinesia or normal, after which patients were randomized in each category to sphincterotomy or a sham procedure in a prospective double blind study. In the SO stenosis group symptoms improved in 11/13 patients treated with sphincterotomy compared to 5/13 who had a sham procedure (P = 0.041). Results from each treatment group did not differ for patients with SO dyskinesia and normal SOM. This trial provided further evidence that patients with presumed SO dysfunction, with subsequent manometrically diagnosed SOD, benefit from endoscopic sphincterotomy. The authors hypothesized a generalized motility disorder to account for the lack of benefit in patients with normotensive but dyskinetic sphincter function.

Sherman *et al*[71] reported results of a randomized trial comparing sphincterotomy, surgical biliary sphincteroplasty with pancreatic septoplasty (with or without cholecystectomy) to sham sphincterotomy for types Ⅱ and Ⅲ biliary patients with manometrically documented SOD (n = 52). After 3 years, 69% of patients undergoing endoscopic or surgical sphincterotomy had symptomatic improvement compared to 24% in the sham sphincterotomy group (P = 0.009). Type Ⅱ patients had an 81% response to sphincter ablation compared to 58% for type Ⅲ patients; double that of the sham sphincterotomy group.

These trials suggest that SOM is a useful guide in predicting benefit from sphincterotomy in type Ⅱ SOD. However, other (non-randomized) trials have suggested that manometric findings do not correlate with clinical outcome. For example, Botoman *et al*[72] included types Ⅱ (n = 35) and Ⅲ (n = 38) patients to assess response to sphincterotomy. There was no difference between the two groups with respect to sphincter hypertension (60% *vs* 55% respectively), symptomatic improvement at 3 years (60% *vs* 56%) or post-procedure pancreatitis rates (15% *vs* 16%). The authors suggested that current classifications are inadequate to define either incidence of SOD or response to sphincterotomy. In another trial SOM was performed in all but 3 patients from a total of 35 patients with suspected type Ⅱ SOD and 29 with type Ⅲ[73]. Sphincterotomy was performed in all patients with SO pressure greater than 40 mmHg, which included 62.5% of the type Ⅱ patients and 50% of the type Ⅲ patients. After 6 wk 70% of the patients with type Ⅱ SOD and 39% of the type Ⅲ SOD who had sphincterotomy reported benefit (P = 0.13, type Ⅱ *vs* type Ⅲ). None of the patients with normal manometry had symptomatic improvement. After long-term follow up (median 2.5 years) sustained improvement occurred in 60% of the type Ⅱ patients but only 8% of those with type Ⅲ (P < 0.01). The investigators felt that the current classification helps predict outcome after sphincterotomy but again acknowledged a lack of difference in the incidence of abnormal SO baseline pressure between type Ⅱ and type Ⅲ SOD.

Cicala *et al*[74] performed SOM and quantitative scintigraphy in 30 patients with suspected type Ⅰ or type Ⅱ SOD. Fourteen (6 type Ⅰ and 8 type Ⅱ) of the 22 patients were offered and underwent sphincterotomy. At long term follow up, all 14 patients were asymptomatic, biochemical abnormalities had resolved and hepatic hilum-duodenum transit time (HHDT) at scintigraphy had significantly decreased. The patients who had refused sphincterotomy had no change in symptoms or HHDT. Scintigraphy predicted favorable outcomes in 93% of cases compared to 57% for SOM. Two other studies found no correlation between response to sphincterotomy and sphincter pressure for either type Ⅰ or type Ⅱ patients[68,75].

The frequency of hypertension in either sphincter among patients with presumptive type Ⅲ SOD ranges from 25%-70%[76]. The previously cited trial by Sherman *et al*[71], which was published as an abstract, is the only randomized controlled trial that has dealt with outcomes post-sphincterotomy for patients with type Ⅲ SOD. 29 patients with presumed type Ⅲ SOD were randomized and after a 3-year follow up period symptoms had improved in 8/13 (62%) who had undergone endoscopic sphincterotomy, 3/10 (30%) who has sham sphincterotomy and 3/6 (50%) after surgery. A follow up study after dual sphincterotomy for biliary and pancreatic SOD, which included 166 patients with type Ⅲ SOD, found no significant difference in re-intervention rates between different classes of SOD (i.e., biliary *vs* pancreatic, type Ⅱ *vs* type Ⅲ)[77]. After a mean follow up of 44 mo, persistent symptoms prompted re-intervention in 28.3% of patients with type Ⅲ SOD, compared to 20.4% for combined type Ⅰ and Ⅱ (P = 0.105). Other studies report response rates between 8%-65% for type Ⅲ SOD[73,76].

It has been postulated that type Ⅲ SOD is part of a spectrum of functional GI disorders and many patients labeled with it may in fact have a diffuse gastrointestinal motility disturbance. Desautels *et al*[78], for example,

showed that patients with type Ⅲ SOD exhibit duodenal-specific visceral hyperalgesia and their symptoms are reproduced by duodenal distension. The challenge remains to identify which patients will most likely to benefit from a particular therapy. Varadarajulu *et al*[2] suggest that patients who present with discrete, self-limiting episodes of typical biliary or pancreatic type pain are the ones most likely to benefit from SOM and sphincterotomy. With the current evidence available it is reasonable to consider medical therapy as the first line of treatment for patients with suspected type Ⅲ SOD. ERCP with SOM should be considered in the event of failure of medical therapy with sphincterotomy if manometry is abnormal.

Pancreatic type SOD

Evidence that SOD may be a cause of IARP is supported by the resolution of pancreatitis after sphincterotomy, with up to 80% improvement in patients with IARP after biliary sphincterotomy[79]. Tarnasky *et al*[80] showed that biliary sphincterotomy reduced pancreatic basal pressure to within the normal range in 30% of patients immediately after the procedure and 20% after longer term follow up, presumably by ablation of the common channel sphincter, and hence a reduction in the length of the residual pancreatic portion. In a proportion of patients therefore, biliary sphincterotomy alone may resolve pancreatitis or pancreatic pain.

In the one controlled trial addressing response to therapy in patients with acute recurrent pancreatitis presumed to be secondary to SOD, Jacob *et al*[81] compared response to ERCP with or without stent insertion in patients with negative investigations including SOM. Stent insertion reduced the rate of recurrence of pancreatitis from 53% to 11% over a 3-year study period.

Kaw *et al*[54] assessed the relationship between microlithiasis and sphincter hypertension in 67 patients with IARP. After endoscopic biliary sphincterotomy, 88% of patients with type I SOD and 73% with type Ⅱ were asymptomatic, irrespective of microlithiasis or gallbladder status. In a study in which ERCP, SOM and endoscopic ultrasound (EUS) were carried out on 90 patients with acute recurrent pancreatitis, SOD was found to be the most common cause found ($n = 28$) [53]. Of the 22 of these patients who underwent biliary sphincterotomy 21 had reduced episodes of acute pancreatitis after 6 mo.

It has been suggested that inadequate pain relief after biliary sphincterotomy may be due to inadequate biliary sphincterotomy, recurrent biliary stenosis, chronic pancreatitis, other residual pancreaticobiliary disease or a non-pancreaticobiliary cause, e.g., irritable bowel syndrome or a persistent abnormality in pancreatic sphincter pressure[28,82,83]. In the latter case, dual biliary and pancreatic sphincterotomy may improve outcome. Eversman *et al*[84] reported long term outcome of biliary sphincterotomy alone in patients with SOD. Patients with SOD and an abnormal pancreatic sphincter pressure needed re-intervention more often than those with abnormal biliary sphincter pressure alone (39.4% *vs* 16.2%, $P < 0.05$) or dual sphincter hypertension (29%, $P < 0.05$). These results support the theory that an untreated pancreatic SOD may cause recurrent pain in patients who have undergone biliary sphincterotomy alone. A previously cited study by the same authors[16] showed that manometry of both pancreatic and biliary portions of the SO is necessary for complete evaluation for SOD. Other studies have drawn the same conclusions[22,85].

Guelrud *et al*[86] reported the response to four different therapeutic options in patients with normal pancreatography and elevated sphincter pressures (pancreatic type Ⅱ SOD). Symptomatic improvement occurred in 28% of patients treated by biliary sphincterotomy alone, in 54% who had biliary sphincterotomy combined with pancreatic orifice dilatation, in 77% who underwent dual sphincterotomies at two separate sessions and in 86% of patients who had dual sphincterotomies performed during a single session. Compared to biliary sphincterotomy alone, dual sphincterotomy had significantly better outcomes ($P < 0.0005$), irrespective of whether they were performed at a single or at separate sessions. The authors suggested that pancreatic sphincter ablation should be considered for patients with type Ⅱ SOD and an abnormal pancreatic basal sphincter pressure. Other studies have shown similar results. Soffer and Johlin[87] found symptomatic improvement following pancreatic sphincterotomy in 16 out of 25 (64%) patients unresponsive to biliary sphincterotomy. In a further trial, 43 patients who had not responded to biliary sphincterotomy were followed up for a median of 14 mo after pancreatic sphincterotomy. 39/43 patients (91%) showed clinical improvement with 31/43 having a complete response[88].

Another group of investigators followed-up 313 patients who had undergone endoscopic dual sphincterotomy for manometry documented SOD of at least one sphincter for a mean of 43.1 mo[77]. Hypertension was demonstrated in both sphincters in 57%, in the pancreatic sphincter alone in 35% and in the biliary sphincter alone in 26%. Immediate complications occurred in 15% of patients and re-intervention was required in 24.6% of patients at a median follow-up of 8 mo. Re-intervention rates were similar irrespective of ducts with abnormal basal sphincter pressure or previous cholecystectomy. Compared to biliary sphincterotomy alone in historical controls, dual sphincterotomy had a lower re-intervention rate in patients with pancreatic SOD alone (21.3% *vs* 39.4%, $P = 0.034$) and a comparable outcome in those with SOD of both ducts (26.6% *vs* 29%, $P = 0.412$) or isolated biliary SOD (25% *vs* 16.2%, $P = 0.285$). Immediate complication rates occurred in 47/313 patients (15%) with pancreatitis in 45/313 (14.4%). Severe pancreatitis occurred in 0.9% of patients. These complication rates are lower than those reported for biliary sphincterotomy in the prospective study by Freeman *et al*[36] when 21.7% of patients developed pancreatitis, of which 3.7% were severe. This may relate to differences in the quality of pancreatic drainage between the two trials. Fogel *et al*[26] also noted that biliary, as opposed to dual, sphincterotomy was more likely to induce pancreatitis in patients with suspected SOD. Therefore, dual sphincterotomy seems to be beneficial for patients with pancreatic SOD, but not in those with biliary SOD alone. It remains unclear

whether dual sphincterotomy should be performed at the initial procedure. Further randomized trials comparing single versus dual sphincterotomy in patients with SOD are necessary to determine the most appropriate sphincter therapy based on SOM findings. However, other factors should also be taken into account. In a recent trial which included patients with biliary types Ⅰ, Ⅱ and Ⅲ SOD, all 121 patients underwent biliary sphincterotomy[89] and 49 patients had pancreatic sphincterotomy at initial or subsequent ERCP if there was a history of abnormal pancreatic manometry in the setting of continuous pain, persistent pain after biliary sphincterotomy or a history of amylase elevation. There was no significant difference in patient response according to Milwaukee classification. (However, this may reflect the numbers of patients involved, with only 18 meeting the criteria for type Ⅰ SOD). Significant predictors of poor response were normal pancreatic manometry, delayed gastric emptying, daily opioid use and age < 40. Abnormal liver function tests and a dilated bile duct were not significant predictors of outcome. These findings support the argument that we cannot rely on the Milwaukee classification alone to predict response to treatment. The authors suggested that patient factors and pancreatic manometry may be more important predictors of outcome of dual sphincterotomy for SOD. These issues should be taken into account before embarking on therapy.

POST-ERCP PANCREATITIS IN SOD

Overall pancreatitis rates post- ERCP are usually quoted to be between 5%-15%[36,90]. Prospective studies have consistently shown that SOD confers increased risk of post-ERCP pancreatitis (PEP). Cheng *et al*[38] evaluated risk factors for ERCP-induced pancreatitis in 1115 patients who had undergone ERCP. Suspected SOD was a significant risk factor with an OR of 2.6. In a prospective study of 1223 ERCP procedures, Vandervoort *et al*[91] found that patients with manometrically proven SOD had a threefold risk of PEP (21.7% *vs* 7.2%). Freeman *et al*[92] found an overall pancreatitis rate of 6.7% in 1963 ERCP procedures with an odds ratio of 2.6 for suspected SOD. A meta-analysis of 15 prospective clinical trials found that patients with suspected SOD had a relative risk of developing pancreatitis of 4.09 (95% CI 3.37-4.96, $P < 0.001$)[93]. SOD is therefore an independent risk factor for post-ERCP acute pancreatitis and the decision to proceed to ERCP, with or without SOM and/or sphincterotomy, should be made with care.

Sphincterotomy for SOD increases the risk of PEP. One randomized control trial, albeit small ($n = 36$), found a post-sphincterotomy pancreatitis rate of 33% in patients with SOD in whom a PD stent was not placed[94]. Five prospective randomized trials have compared PEP rates between high risk patients with or without PD stent placement. Four of these included patients with SOD (Table 3)[25,35,94,95]. Of these four studies all showed a trend to reduction of PEP with PD stent placement, and two reached statistical significance. A meta-analysis of five prospective studies showed a 3-fold increased risk of post-ERCP pancreatitis if a pancreatic stent was not used (15.5% *vs* 5.8%, OR 3.2, 95% CI 1.6-6.4)[96].

Table 3 Role of pancreatic stent insertion in prevention of post- ERCP pancreatitis; results of randomized controlled trials that included patients with SOD

Author	Year	No. of patients	Pancreatitis rate (%)		
			Stent	No stent	
Smithline *et al*[95]	1993	93	14	18	$P = 0.299$
Tarnasky *et al*[25]	1998	80	7	26	$P = 0.03$
Patel *et al*[94]	1999	36	11	33	$P > 0.05$
Fazel *et al*[35]	2003	76	5	28	$P < 0.05$

At least three case control studies have also included patients with SOD. In two of these there was a significant reduction in PEP with a pancreatic stent[26,88] and in the other the reduction of pancreatitis rate from 66.7% to 14.4% did not quite reach significance ($P = 0.06$)[97]. Therefore, there is substantial evidence that pancreatic stent placement reduces the incidence of post-ERCP pancreatitis in high-risk groups such as SOD. However, failure to deploy the stent successfully may occur in up to 10% of patients[98], and failed pancreatic stent placement can increase the rate of PEP sixteen fold[97]. Therefore, pancreatic stent placement should not be attempted unless the likelihood of success is very high.

CONCLUSION

The relationship between SOD and pancreatitis is a complex one. An association between SOD and acute pancreatitis appears to be beyond doubt, not least because of the high frequency of abnormal SOM in IARP. SOD also carries a significantly increased risk of post-ERCP pancreatitis with rates of over 30%, although correct placement of a pancreatic stent at the time of the procedure appears to reduce this risk. However, although various mechanisms have been postulated, the exact role of SOD in the pathophysiology of pancreatitis is not known and it is unclear if SO dysfunction as a primary event or secondary to other factors is the principal mechanism. There is also evidence linking SOD with chronic pancreatitis but whether this is a cause or effect relationship is still unknown.

Sphincterotomy remains the management of choice for SOD. All patients with type Ⅰ SOD should have their sphincter ablated and, by general consensus, this group does not require manometry prior to the procedure. The question whether dual sphincterotomies should be carried out remains unanswered and further randomized trials are required to clarify this. For patients with type Ⅱ SOD grade A studies have found that SOM is a useful guide in predicting response to sphincterotomy, although some smaller studies showed that manometric findings do not correlate with clinical outcome. However, most experts agree that patients with suspected type Ⅱ SOD should have SOM before considering sphincterotomy.

The management of patients with type Ⅲ SOD is more difficult still with response rates to sphincterotomy ranging from 8% to 65%. In general, sphincter ablation is probably warranted if SOM is abnormal but medical

therapy should be tried before proceeding to manometry. Detailed history taking is paramount for these patients. The more the pain pattern differs from that set out in the Rome Ⅱ criteria, the less likely is the patient to benefit from treatment. Until there is a more adequate method of characterizing patients with type Ⅲ SOD it will not be possible to carry out a randomized trial of sphincterotomy against placebo. Ultimately, this will be the only way of proving the benefit or otherwise of sphincterotomy for patients with presumptive type Ⅲ SOD.

Recent evidence supports the need to measure both portions of the SO to maximize the detection rate of SOD. This dual classification has prompted a call for a single overall classification system from some quarters. A recently published trial[89] has also shown that other patient factors such as age, opioid use, delayed gastric emptying and pancreatic manometry are more important predictors of response to dual sphincterotomy than abnormal liver function tests and a dilated ductal system, on which the traditional classification system is heavily based. Further large prospective trials are required to identify other potential patient factors that may help predict response to therapy; such factors should be taken into account in any future overhaul of the current classification system.

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TOPIC HIGHLIGHT

Michael F Byrne, MD, *Series Editor*

Pancreatic endocrine and exocrine changes in celiac disease

Hugh J Freeman

Hugh J Freeman, Department of Medicine (Gastroenterology), University of British Columbia, Vancouver, BC, Canada
Correspondence to: Dr. Hugh J Freeman, MD, FRCPC, FACP, Gastroenterology, UBC Hospital, 2211 Wesbrook Mall, Vancouver, BC, V6T 1W8, Canada. hugfree@shaw.ca
Telephone: +1-604-8227216 Fax: +1-604-8227236
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Abstract

Although there is a great deal of information on celiac disease and associated involvement of other non-intestinal sites, data on concomitant changes in the structure and function of the pancreas is limited. The present review critically examines pancreatic endocrine changes that have been well documented in the literature, including insulin-dependent diabetes mellitus. Pancreatic exocrine alterations may also occur, and if severe, marked malnutrition with pancreatic failure and ductal calcification have been observed. Finally, other pancreatic disorders have been recorded with celiac disease.

Key words: Celiac disease; Gluten-sensitive enteropathy; Diabetes mellitus; Chronic pancreatitis; Pancreatic function

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INTRODUCTION

Considerable information has already been published on both the intestinal and the extra-intestinal manifestations of celiac disease. Pancreatic changes may be caused by celiac disease or co-exist with celiac disease. Both endocrine and exocrine function of the pancreas may be substantially changed in celiac disease. As a result, superimposed or more severe clinical changes may appear and marked nutritional disturbances may result. Although data is very limited, there is increasing evidence that impaired endocrine and exocrine pancreatic function in celiac disease may be favorably influenced by gluten-free diet treatment.

PANCREATIC ENDOCRINE CHANGES

Over the past decade or so, a number of studies from Europe[1,2] and North America[3,4] have demonstrated that the prevalence of celiac disease in patients with type 1 diabetes is increased. This association is due, at least in part, to sharing of the human leukocyte antigen allele, DR3, and, by linkage disequilibrium, DQ2[5]. In addition to a common "autoimmune" basis, it is conceivable that some celiacs have developed diabetic changes secondary to severe pancreatic insufficiency with exocrine dysfunction. Earlier serological studies employed IgA antibody studies for celiac screening, particularly using IgA endomysial antibody (EMA) testing. This eventually became the "gold standard" for serological studies, until subsequent identification of tissue transglutaminase (TTG) as the tissue antigen for endomysial antibodies[6]. As a result, IgA TTG antibodies (using ELISA methodology) became a very attractive alternative for first line screening and also permitted development of a quantitative assay.

A recent TTG prospective study[7] in children and adolescents with type 1 diabetes evaluated TTG antibody titres in 125 males and 108 females followed in a pediatric diabetes center. Altogether, 26 patients, including 15 males and 11 females, had positive TTG titres and, of these, 19 were also positive for EMA. In those positive for both TTG and EMA, small intestinal biopsies were done. Histopathological abnormalities described in celiac disease were detected ranging from increased numbers of intraepithelial lymphocytes to severe crypt hyperplastic villous atrophy (Marsh 3 lesion)[7]. These studies also suggested that serial TTG serological measurements in insulin-dependent diabetics might play a role in monitoring their serological responses as well as the compliance to the gluten-free diet. In children and adolescents, close monitoring is critical as compliance in these age groups may be especially difficult to assess. While over 40% of the diabetics in this study were asymptomatic[7], prospective serological screening appeared to facilitate selection for biopsy evaluation.

Earlier detection of celiac disease has been urged in type 1 diabetes, even in children, because of the long-term risks of undiagnosed celiac disease. It has been suggested that the longer the duration of untreated celiac disease (also in dermatitis herpetiformis), the higher the risk of

enteropathy-associated T cell lymphoma[8]. Moreover, adherence to a gluten-free diet appears to reduce the risk of enteropathy-associated T cell lymphomas in celiac disease. Finally, the association of lymphoma, celiac disease and type 1 diabetes has also been documented in 4 cases[9].

Other long-term complications may occur including iron deficiency anemia, osteoporosis, infertility and growth retardation. These appear to be most significant when patients are poorly compliant with a gluten-free diet, or if diagnosis is delayed until later in life[10]. Finally, improved glucose control with a gluten-free diet has also been shown in type 1 diabetes with concomitant celiac disease[11]. In these studies with type 1 diabetes in celiac disease, pancreatic exocrine function was not evaluated.

PANCREATIC EXOCRINE CHANGES

Pancreatic exocrine function may also be substantially altered in celiac disease. Even though the precise prevalence of altered pancreatic function in celiac disease is not known, impaired pancreatic function may be a cause of impaired digestion and absorption resulting in malnutrition[12]. It has been estimated that over 20% of patients with celiac disease have defective exocrine pancreatic function[13]. This may be related to several factors. First, impaired secretion and/or release of pancreatic stimulating hormones from the diseased proximal small intestine may be important[14]. Immunohistochemical studies on small intestinal biopsies from untreated celiac disease have demonstrated significant alterations in enteric endocrine cells, including an absence of secretin cells[15].

Moreover, studies with test meals in celiacs have suggested impaired secretion of cholecystokinin-pancreozymin, resulting in reduced pancreatic exocrine cell stimulation[16]. Second, deficiencies of amino acids may result from impaired small intestinal amino acid uptake, leading to reduction in precursors of pancreatic enzyme synthesis[12,17]. Third, protein malnutrition may lead to structural changes in the pancreas, including atrophy of acinar cells and pancreatic fibrosis, resulting in impaired pancreatic exocrine function[12]. Pancreatic enzyme measurements were also found to be reduced with mucosal atrophy and could be inversely correlated with the degree of intestinal damage[18]. Earlier studies measuring xylose absorption were limited in ability to separate pancreatic and intestinal causes of steatorrhea, especially if the degree of impairment was mild[19]. Subsequent attempts to define altered intestinal permeability using large molecules (e.g., cellulobiose, mannitol)[20] or separate pancreatic insufficiency from intestinal dysfunction with noninvasive stable isotopes[21] have not become widely accepted.

Severe structural changes have also been documented. Pancreatic calcification, most often associated with chronic or persisting pancreatic inflammation, has been traditionally associated with excessive alcohol use.

Atrophy, fibrosis and altered pancreatic function have also been detected in experimental animals treated with diets deficient in protein, in adults with protein energy malnutrition, in children with kwashiorkor and in some early autopsy studies of patients with celiac disease[16,17,22-24]. In addition, pancreatic calcification has been reported with chronic protein malnutrition in the Indian subcontinent and in some African countries[25]. Finally, an adult patient with celiac disease and non-alcohol related pancreatitis with calcification has been described in North America[26]. Possible effects on pancreatic exocrine function in celiac disease poorly compliant to a gluten-free diet has not been evaluated.

MISCELLANEOUS DISORDERS

A number of other miscellaneous pancreatic disorders have been associated with celiac disease. Pancreatic mucinous adenomas have been associated with celiac disease in a patient with polycystic kidney disease[27]. In addition, pancreatic and ampullary carcinomas have been recorded in celiac disease[28,29], although the celiac disease has often only been recognized after pancreatoduodenectomy suggesting that subclinical celiac disease may be "unmasked" by major upper gastrointestinal surgery and should be considered in the differential diagnosis after pancreatoduodenectomy[22,29].

Celiac disease has been associated with chronic calcific pancreatitis in patients with pancreas divisum[30]. Diabetes-related autoantibodies may appear in children with celiac disease, including antibodies to insulin, immunoglobulin, islet cells and glucagon[31]. Early-onset vitamin B12 malabsorption in children with celiac disease has also been documented and related to impaired pancreatic exocrine secretion[32]. Finally, macroamylasemia has also been attributed to gluten-related amylase autoantibodies (particularly in childhood)[33].

Because of the possible deleterious effects of biliary tract lithiasis on pancreatic structure and function, gallbladder function in celiac disease may also be important and carefully examined in celiac disease. In some, slow emptying of the gallbladder has been documented, associated with impaired contraction response to fat[34,35]. Studies of enteric endocrine cells have demonstrated significant quantitative and qualitative changes in celiacs[15]. In addition, studies with test meals have suggested impaired secretion of cholecystokinin in patients with celiac disease, and, possibly, impaired gallbladder responsiveness to cholecystokinin[15,34]. In spite of these physiological alterations, there does not appear to be a significant predisposition to gallstones in celiac disease and consequently, secondary pancreatic damage. Only 9 of 350 celiac patients had a cholecystectomy for gallstone disease[14]; however, in a survey of elderly celiacs initially diagnosed after the age of 60 years, approximately 20% had developed gallstone disease[36,37].

CONCLUSION

In summary the endocrine and exocrine function of the pancreas may be impaired in celiac disease and their pathogenesis may be closely linked. Further studies are needed to explore the importance of detection of endocrine and exocrine pancreatic disorders in celiac disease, along with potential treatment options.

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TOPIC HIGHLIGHT

Michael F Byrne, MD, *Series Editor*

Transplantation for the treatment of type 1 diabetes

R Mark Meloche

R Mark Meloche, Department of Surgery, University of British Columbia, Vancouver, British Columbia, Canada
Correspondence to: R Mark Meloche, MD, FRCSC, Department of Surgery, University of British Columbia, 5th Floor Diamond Centre, 2775 Laurel Street, Vancouver, BC, V5Z 1M9, Canada. meloche@interchange.ubc.ca
Telephone: +1-604-8755287
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Abstract

Transplantation of pancreatic tissue, as either the intact whole pancreas or isolated pancreatic islets has become a clinical option to be considered in the treatment of patients with type 1 insulin-dependant diabetes mellitus. A successful whole pancreas or islet transplant offers the advantages of attaining normal or near normal blood glucose control and normal hemoglobin A1c levels without the risks of severe hypoglycemia associate with intensive insulin therapy. Both forms of transplants are also effective at eliminating the occurrence of significant hypoglycemic events (even with only partial islet function evident). Whereas whole pancreas transplantation has also been shown to be very effective at maintaining a euglycemic state over a sustained period of time, thus providing an opportunity for a recipient to benefit from improvement of their blood glucose control, it is associated with a significant risk of surgical and post-operative complications. Islet transplantation is attractive as a less invasive alternative to whole pancreas transplant and offers the future promise of immunosuppression-free transplantation through pre-transplant culture. Islet transplantation however, may not always achieve the sustained level of tight glucose control necessary for reducing the risk of secondary diabetic complications and exposes the patient to the adverse effects of immunosuppression. Although recent advances have led to an increased rate of obtaining insulin-independence following islet transplantation, further developments are needed to improve the long-term viability and function of the graft to maintain improved glucose control over time.

Key words: Type 1 diabetes; Insulin-dependant diabetes mellitus; Pancreas transplantation; Pancreatic islet transplantation; Immunosuppression; Glucose control

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TRANSPLANTATION FOR THE TREATMENT OF TYPE 1 DIABETES

It has been clearly shown that patients with type 1 insulin-dependent diabetes mellitus (IDDM) benefit from improved blood glucose control. In 1993, the Diabetes Control and Complications Trial Research Group reported that patients with IDDM treated with intensive insulin therapy showed a reduced risk of developing retinopathy, albuminuria or microalbuminuria and clinical neuropathy when compared to patients who received conventional insulin therapy[1]. In this trial the intensive therapy group was shown to have achieved sustained lowered blood glucose concentrations over time as reflected by significantly lower glycosylated hemoglobin values compared to those of the conventional insulin therapy group. Although the intensive therapy group benefited from reduced long-term complications, the risk of severe hypoglycemia associated with tight glycemic control was three times greater than in the conventional therapy group.

Transplantation of pancreatic tissue, as either the intact whole pancreas or isolated pancreatic islets has become a clinical option to be considered in the treatment of patients with IDDM. Successful pancreatic transplantation offers the advantages of attaining normal or near normal blood glucose control without the risks of severe hypoglycemia associated with intensive insulin therapy. Pancreatic transplantation however, may not always achieve the sustained level of tight glucose control necessary for reducing the risk of secondary diabetic complications and exposes the patient to peri-operative or procedural risks, the adverse effects of immunosuppression and the risk of an eventual significant loss of graft function necessitating a return to exogenous insulin.

When either transplantation of a whole pancreas or pancreatic islets is being offered for the treatment of IDDM, the goals of the program should include: (1) A transplant procedure, which can be performed with overall low morbidity and mortality and subject the recipient to a minimal degree of side effects and complications of the post-procedural care; (2) Elimination of the need for insulin administration and close blood glucose monitoring; (3) Eliminate the occurrence of significant hypoglycemic events; (4) Creation of a euglycemic state, with preprandial and postprandial blood glucose concentrations and

hemoglobin A1c levels comparable to those in the non-diabetic population; (5) Achieving sustained effect of the transplant as to maintain normal glucose homeostasis over time.

WHOLE PANCREAS TRANSPLANTATION

Whole pancreas transplantation, first performed in 1966 in combination with a kidney in IDDM patients suffering from end-stage renal failure, demonstrated that a euglycemic state could be obtained without the need for exogenous insulin[2]. The early procedures however, were complicated by a high morbidity rate; early graft failure and poor patient survival and few transplants were performed[3]. Improvements in transplant techniques, immunosuppression therapies and post-transplant monitoring of graft function and rejection has resulted in a dramatic improvement in patient morbidity and graft survival. With the improved outcomes and demonstrated efficaciousness in controlling the diabetic glycemic state, whole pancreas transplantation is now recognized by the American Diabetes Association as an acceptable therapeutic alternative to continued insulin therapy in diabetic patients with imminent or established end-stage renal disease who had or plan to have a kidney transplant[4].

Pancreas transplantation may be considered as a group of three separate, clinical entities: simultaneous pancreas and kidney transplant (SPK), pancreas after kidney transplant (PAK) and pancreas transplant alone (PTA). Each form of transplant is characterized by its own indications, risks and outcomes.

Simultaneous pancreas kidney transplantation

Diabetes is a major cause of renal disease and is associated with approximately 40% of new cases of end-stage renal failure (ESRF) in the US, who will subsequently require renal dialysis or kidney transplantation[5]. When an IDDM patient develops ESRF and requires a kidney transplant; consideration is now commonly given to whether the patient would also benefit from receiving a pancreas transplant. SPK transplantation is the most common form of pancreas transplantation performed accounting for 60% of the total number of pancreas transplants performed each year in the US (approximately 900/year)[6-8]. The annual number of SPK transplants has remained stable over the last 10 years, however this may reflect the increasing interest in the option of PAK transplantation (living donor kidney transplantation) by the patient in ESRF with IDDM.

When SPK transplantation is performed, the patient undergoes only one operation and following the surgery may be managed on the same (or very similar) immunosuppressive drugs they would have received for a renal transplant alone. The combined procedure offers excellent patient and pancreatic graft survival, producing a sustained euglycemic state off of exogenous insulin or oral hyperglycemic agents[7,9]. The renal transplant provides an effective method of surveillance of both grafts for acute rejection (creatinine clearance, biopsy) and may in part explain the improved one-year and long-term pancreatic graft survival rates when compared to a pancreas transplant without a donor-matched kidney transplant. There also has been no evidence that the pancreas transplant may have a deleterious effect on the simultaneously transplanted kidney.

Pancreas after kidney transplantation

As the number of patients with ESRF increased in the past decade, the demand for renal transplantation outpaced the supply of cadaveric kidneys available. By 2000, the number of living donor kidney transplants performed in the US exceeded the number of cadaveric donor transplants. Whereas SPK transplantation is usually restricted to the use of cadaveric kidneys only, PAK transplantation offers the option of using a living donor kidney, and in doing so both expands the number of kidneys available for transplant and allows the diabetic patient the opportunity to benefit from early living donor kidney transplant (better long term outcome, avoidance of dialysis). With improved surgical technique, better immunosuppressive drugs and rejection monitoring, outcomes for solitary pancreas transplants have improved such that PAK transplantation is now routinely considered[6,7]. The kidney transplant recipient is required to demonstrate stable renal function and minimal post procedure complications to be acceptable for subsequent pancreas transplantation. The PAK transplant option does require the patient to undergo two major operations, however the post transplant immunosuppression and care are similar to kidney transplant alone.

Pancreas transplantation alone

PTA is offered in some transplant centers to IDDM patients who have difficult to manage diabetes and suffer from severe hypoglycemic episodes (often with hypoglycemic unawareness) with little or no evidence of renal disease. As with PAK transplantation, improvements in PTA graft survival has significantly improved in recent years, although the question of whether or not the procedure may have an adverse long term affect on the recipients renal function (calcineurin antagonist based immunosuppression) has not been resolved. The normalizing of blood glucose levels with the pancreas transplant may offer this patient group the possibility of long-term improvement in renal function. The American Diabetes Association's 2006 Position Statement on pancreas and islet transplantation recommends "In the absence of indications for kidney transplantation, pancreas-alone transplantation should only be considered a therapy in patients who exhibit these three criteria: (1) a history of frequent, acute and severe metabolic complications (hypoglycemia, marked hyperglycemia, ketoacidosis) requiring medical attention; (2) clinical and emotional problems with exogenous insulin therapy that are so severe as to be incapacitating; and (3) consistent failure of insulin-based management to prevent acute complications"[4]. Since 2001, PTA has accounted for approximately 12% of pancreas transplants performed annually in the US[6].

WHOLE PANCREAS TRANSPLANTATION SURGERY

Bladder verses enteric pancreatic drainage

In the early development of pancreas transplantation, the surgical procedure involved drainage of the exocrine pancreas secretions in to the bladder. This was best accomplished by transplanting (in continuity) a short segment of donor duodenum with the pancreas, which provides a conduit from the pancreas to the bladder through which the secretions could be drained. Use of the duodenum-to-bladder drainage reduced the rate of transplant exocrine secretion leaks and allowed monitoring for evidence of early graft rejection by the frequent measurement of amylase activity in the urine. Direct biopsy of the transplanted pancreas and duodenum by cystoscopic technique could also be performed[3]. However, the loss of bicarbonate-rich pancreatic secretions into the bladder is associated with a number of problems. Patients who have undergone bladder drainage of their pancreas transplant require daily oral bicarbonate and fluid replacement to offset the severe metabolic acidosis and at times significant fluid loss associated with the procedure. Other problems associated with bladder drainage include: reflux graft pancreatitis (bladder dysfunction associated with diabetic neurogenic bladder); cystitis; urethritis and stricture formation; recurrent bladder infections. For some patients (approximately 15% at three years from the time of transplantation) the complications were severe enough that surgical reversal of the bladder drained pancreas transplant, with re-direction of the secretions into the small bowel was necessary[3,9].

In the late nineties transplant centers began to perform primary enteric drainage of pancreatic secretions in order to avoid the complications of bladder drainage. Improvements in immunosuppression had led to a reduced number of episodes of acute graft rejection and increasing experience with direct percutaneous needle biopsy reduced the need for urinary amylase monitoring. Enteric drainage of pancreatic secretions prevented the obligatory loss of sodium bicarbonate and fluid and the subsequent hypovolemia, metabolic acidosis and the other complications associated with bladder drainage. Initially the procedure often involved anastomosis of the transplant duodenal segment to a Roux-en-Y limb of the recipient's mid-small bowel to reduce the likelihood of an anastomotic leak, however the surgery is now routinely performed directly to the small bowel. Enteric drainage of the pancreatic transplant secretions is now the more commonly performed procedure, used in 81% of SPK, 67% of PAK and 56% of PTA transplants performed in the US during 2000-2004 (when a SPK was performed, the donor-matched kidney may be followed for evidence of acute graft rejection which may be a better early marker than urinary amylase output)[6-10].

Systemic verses portal venous drainage

Vascular drainage of the pancreas transplant may be performed either into the systemic venous system (most commonly used is the external iliac vein) or into the portal venous system (using a vein of the small bowel mesentery). With systemic venous drainage the insulin secreted by the pancreas transplant avoids early hepatic extraction and results in an elevation of both basal and stimulated serum insulin concentrations. Portal venous drainage directs the insulin released by the pancreas transplant initially to the liver in a fashion similar to the release of insulin in a non-diabetic person. Although it is felt to be "more physiological", portal venous drainage of the pancreas transplant has not been shown to offer any advantage over systemic venous drainage in maintaining normal glucose homeostasis or lipid metabolism. It has been suggested that direct presentation of the pancreas transplant alloantigen to the liver may provide an immunologic benefit. However, registry data has not found evidence for improved graft survival when portal venous drainage has been performed. Systemic venous drainage remains the more commonly performed procedure, used in 77% of SPK, 73% of PAK and 56% of PTA transplants performed in the US during 2000-2004[6-9].

Surgical complications

The complexity of the whole pancreas transplant procedure, along with the likelihood of pre-existing disease secondary to their IDDM, exposes the recipient to a variety of significant operative and post-operative risks. The extent of the post-operative problems likely limited the widespread acceptance of pancreas transplantation in the early era of its development. Serious surgical complications following the procedure include: thrombosis of graft vessels, intra-abdominal hemorrhage, anastomotic leak (enteric or bladder), graft pancreatitis, pancreatic fistula formation and intra-abdominal sepsis, all of which may require re-laparotomy and the possibility of graft loss. In recent years, with improvements in donor and recipient selection criteria, surgical technique, immunosuppression protocols (reduced incidence of early, acute rejection) and prophylaxis regimes (anti-viral, anti-bacterial and antithrombosis), there has been a significant decrease in the overall incidence of serious complications and the rate of re-laparotomy[11,12].

Immunosuppression

Since 2000, in the US the most common primary protocol for maintenance immunosuppression for whole pancreas transplantation is Tacrolimus and Mycophenolate Mofetil (MMF), although other agents such as Cyclosporine, Sirolimus and Azathioprin in varying combinations are also being used in a small number of centers[7,8]. In addition, the majority of transplant centers continue to use corticosteroids, although there is an movement towards "steroid-free" immunosuppression protocols in an attempt to reduce their adverse effects (glucose control, dyslipidemia, bone loss) in the diabetic patient transplant population. The initial success with pancreatic islet transplantation in 2000 was obtained using an immunosuppression protocol of Sirolimus and Tacrolimus in combination and avoiding steroid entirely[13]. Sirolimus has proven to be difficult to use in some patients (mouth ulcers, hyperlipidemia)[14] and recent studies

have demonstrated poorer graft survival and inferior renal function when Sirolimus is used as a primary agent in combination with Tacrolimus when compared with the use of Tacrolimus and MMF in kidney and heart transplantation[15-17]. Induction immunosuppression is routinely used in whole pancreas transplantation, with > 75% of recipients receiving either: (1) a T-cell depleting polyclonal antibody (Thymoglobulin, ATGAM) or monoclonal antibody (OKT3, Campath), (2) a non-depleting monoclonal anti-CD25 antibody (Zenapax, Simulect), or (3) both[7,8].

Long-term use of immunosuppression is associated with a number of significant side effects and complications. The side effects most commonly seen with standard maintenance immunosuppression include: nephrotoxicity, hypertension, hyperlipidemia, microvascular disease, glucose intolerance, gastrointestinal problems, weight gain, skin changes/alopecia/hirsutism[18]. Immunosuppression-relate complications include: infections (viral, bacterial, fungal, parasitic) and malignancy (skin, lymphoproliferative, genitourinary). The risk of infection depends upon the degree of immune compromise created by the immunosuppressive regiment and the exposure to possible pathogens, either through re-activation of pre-existing infection (e.g., viral, tuberculosis) or introduction by the transplant process (e.g., surgical technique, transfer from donor)[19]. The risk of skin cancer for a patient on immunosuppression following renal transplantation is cumulative, ranging from 10% to 40% at 10 and 20 years respectively (ratio of squamous cell to basal cell carcinoma 2:1)[20]. The overall prevalence of post-transplant lymphoproliferative disease and leukemia varies from 1% to 2%[21].

OUTCOMES OF WHOLE PANCREAS TRANSPLANTATION

Patient and graft survival

Whole pancreas transplantation has proven to be a safe procedure with a 1 year and 3 year patient survival rates for all forms of pancreas transplant (SPK, PAK, PTA) in the US since 1998 at about 95% and 89% respectively (unadjusted patient survival rates, 2005 OPTN/SRTR Annual Report)[6] (Table 1). Transplantation of a pancreas simultaneously with a kidney may also increase long-term IDDM patient survival compared to kidney transplantation alone. Whereas the mortality risk is increased over the first 18 months for the SPK recipient (associated with an increased rate of complications such as infection when compared to kidney transplant alone), when the pancreas continues to function post transplant, recipient survival is superior to SPK recipients who have had their pancreas graft fail or diabetic patients who received only a cadaveric kidney transplant[22,23].

One and 3 year graft survival rates for SPK transplant in the US since 1998 for kidney are ⩾ 91% and ⩾ 83% and pancreas are ⩾ 82% and ⩾ 75% respectively. Pancreas graft survival rates for PAK and PTA over a similar period were slightly less that for SPK: for 1 year 72% to 81% and 74% to 83% respectively, and at 3 years 63% to 71% and 60% to 69% respectively (unadjusted graft survival rates, 2005 OPTN/SRTR Annual Report). Although the incidence of acute kidney graft rejection has been shown to be greater following SPK than for kidney transplant alone (15% *versus* 9%), patients following SPK transplantation as a group general demonstrate better kidney graft function. This advantage of SPK on renal function disappears however when the analyses are adjusted for donor and recipient variables[9].

Table 1 Unadjusted patient and graft survival following SPK, PAK or PTA by year of transplant at 1 and 3 years

			Year of transplant					
		Survival (%)	1998	1999	2000	2001	2002	2003
SPK	Patient	One year	93.9	94.8	95.1	94.1	94.8	95.5
		Three years	89.6	90.6	90.2	89.6	+	+
PAK	Patient	One year	94.5	94.4	96.0	95.2	95.7	95.5
		Three years	90.8	88.1	91.2	89.1	+	+
PTA	Patient	One year	96.8	97.3	99.1	97.5	97.6	94.5
		Three years	90.5	90.9	95.5	90.8	+	+
SPK	Pancreas	One year	82.7	83.1	84.0	85.0	85.4	85.8
		Three years	75.8	76.0	77.0	78.9	+	+
	Kidney	One year	91.2	91.5	92.5	91.5	91.2	91.7
		Three years	83.9	83.0	83.5	83.7	+	+
PAK	Pancreas	One year	72.0	80.3	74.0	81.8	77.3	77.9
		Three years	63.4	66.8	62.3	71.5	+	+
PTA	Pancreas	One year	79.2	83.3	75.3	78.3	79.3	74.4
		Three years	60.3	69.3	60.5	64.4	+	+

Source: 2005 OPTN/SRTR annual report.

CONSEQUENCES OF WHOLE PANCREAS TRANSPLANTATION

Blood glucose control

Successful whole pancreas transplantation produces a normoglycemic state in the majority of recipients, usually within minutes of completion of the procedure without the need for exogenous insulin. Transient hypoglycemia may occur over the first 24 h requiring I.V. glucose support. Patients demonstrate normal fasting and post-prandial blood glucose concentrations and a lowering of hemoglobin A1c to normal levels. Where systemic venous drainage of the pancreas has been performed, fasting and meal-stimulated insulin concentrations are elevate, the likely result of the elimination of first-pass hepatic extraction. Portal venous drainage typically results in a more normal pattern of fasting and meal-stimulated insulin concentrations, with similar glucose control. Although insulin levels are elevated by systemic venous drainage, blood glucose homeostasis appears to be unaffected, demonstrating normal glucose utilization and hepatic glucose production. Whole pancreatic transplantation is also an effective treatment for patients who had a long history of severe, symptomatic hypoglycemia. The normal glucagon response to hypoglycemia is restored and hypoglycemic episodes are uncommon. Whole pancreas transplantation has been shown to be effective in providing recipients with long-term normal glycemic control off insulin (10 years or more). Reduced hemoglobin A1c levels are maintained and patients demonstrate fasting

blood glucose and glycemic control in response to a meal or glucose challenge similar to those of the non-diabetic population[9,24].

Secondary complications of IDDM

The microvascular, neurologic and macrovascular diseases associated with IDDM has been attributed to long-term poor glycemic control. Whereas the Diabetes Control and Complications Research Group reported that improved glucose control through intensive insulin therapy effectively delayed the onset, or slowed the progression of diabetic retinopathy, nephropathy and neuropathy, the risk of severe hypoglycemia was significant and only a small percentage of patients could sustain the required improvement in metabolic control. Whole pancreas transplantation has now been performed over a long enough period of time to allow study of the effect of sustained normal glycemic control in patients with IDDM.

Diabetic nephropathy

Whole pancreas transplantation does prevent de-novo diabetic changes, which would otherwise occur in a diabetic recipient of a kidney transplant[25]. There is also evidence that long-term successful pancreas transplantation may improve pre-existing histological changes secondary to diabetes in the native kidneys, although the effect is only observed after 5 or more years[26]. Whether native renal function benefits from PTA is uncertain, as the nephrotoxic effect of calcineurin inhibitor based immunosuppression therapy must be considered. Registry data has identified that from 2% to 8% of PTA recipients develop ESRF and require a kidney transplant by one year[9,27]. A recent report of case matched PTA with diabetic controls found however that although native renal function decreased significantly after PTA in patients with decreased creatinine clearance (CrCl $\leqslant$ 70 mL/min) at the time of transplantation, it was well tolerated among patients with a CrCl $\geqslant$ 70 mL/min[28]. Another study also found evidence for improvement of renal function after pancreas transplantation, documented by reduction of urinary excretion of protein with stable creatinine concentration and CrCl[29].

Diabetic retinopathy

The diabetic population undergoing pancreas transplantation typically has already developed some degree of retinal pathology and most have received laser therapy. Advanced retinal change does not seem to benefit from pancreatic transplantation as the damage has already occurred. Initial studies that examined the short-term effect of pancreas transplantation on diabetic retinopathy were unable to demonstrate any positive effect of corrected blood glucose control when compared to diabetic recipients of a kidney alone or SPK with a failed pancreas graft[30]. Studies which followed successful pancreas transplants for 5 or more years however, do show a benefit to the recipient with mild to moderate disease, with stabilization of established retinopathy, delay in the progression of new disease, improvement in visual acuity and a reduction in the use of laser therapy[9,31].

Diabetic neuropathy

Polyneuropathy is a common complication of both IDDM and ESRF and advanced motor, sensory and autonomic neuropathies are frequently seen in patients undergoing whole pancreas transplantation. Improvement of both motor and sensory neuropathies symptoms will occur following kidney transplantation alone, however correction of uremia secondary to diabetic nephropathy by kidney transplantation does not halt the progression of the underlying diabetic neuropathic process. Diabetic patients studied following pancreas transplantation with return to a normoglycemic state do show a significant early improvement in sensory and motor nerve conduction studies that continue to improve over time. However clinical neurological examination and testing for autonomic nerve dysfunction demonstrated little improvement, even when followed over a longer period of time[32-35].

Micro- and macrovascular disease

Diabetic microangiopathy, the result of chronic hyperglycemia and subsequent metabolic disturbances seen in IDDM, is the principle cause of many of the severe late complications of diabetes[36]. The progression of microvascular disease-related problems (e.g., nephropathy, retinopathy neuropathy) is reduced when tight glucose control has been obtained, either through intensive insulin therapy or following successful whole pancreas transplantation. Direct evidence for improvement of the microvasculature following pancreas transplantation can also be demonstrated. Skin blood flow characteristics (as measured by laser Doppler), a measure of the degree of microcirculation impairment, have been shown to improve (but not normalize) following pancreas transplantation when compared to non-diabetic controls[37]. The calcineurin-inhibitors, Cyclosporine and Tacrolimus, in addition to being nephrotoxic, have been shown to produce microangiopathy following transplantation and their use may negate any benefit a diabetic recipient might obtain from tight glucose control[38,39].

There is evidence that whole pancreas transplantation may reduce the risk of macrovascular disease. Carotid intima media thickness (determined by carotid ultrasound), a measure shown to correlate with the likelihood of cardiovascular events in IDDM (coronary artery disease, atherosclerotic vascular disease, mortality), is reduced following whole pancreas transplantation. The reduction of the carotid intima media thickness occurs early following transplantation and is independent of other causative factors such as smoking, age, serum lipid concentration and failure of a kidney transplant[40]. However, the typical recipient of a pancreas transplant has had long-standing IDDM and frequently has established vascular intimal disease and plaque formation and clinically significant vascular disease that may progress following transplantation[41].

Quality of life

Patients who have received a whole pancreas transplant consistently report an improvement in their quality of life (QOL), although pancreas transplantation is a complex

surgical procedure, requires life-long immunosuppression and careful follow-up, and is associated with a significant incidence of complications, including re-hospitalization. The improvement in QOL is maintained unless the pancreas recipient experienced graft loss. For patients with IDDM who are in ESRF, a significant improvement in their health may be expected following either correction of uremia or elimination of their diabetic state. In a prospective, longitudinal study of patients undergoing either SPK or kidney transplant alone (KTA), Gross *et al* found that the addition of a pancreas transplant increased the measure of QOL beyond the improvement seen with KTA and the pancreas recipients reported greater improvement in areas that are diabetes-specific[9,42-44].

Pancreatic islet transplantation

As discussed above, the Diabetes Control and Complications Trial Research Group reported that improved glycemic control through intensive insulin therapy delays the onset and slows the progression of diabetic complications. Improved glycemic control however, was hard to sustain and associated with intense insulin therapy was a significant increase in severe hypoglycemic episodes. Whole pancreas transplantation is capable of producing a sustained, euglycemic state, reducing the incidences of hypoglycemia and offering the possible benefit of reducing microvascular, macrovascular and neurologic complications. Pancreas transplantation however, is a major, complex surgical procedure associated with significant risk and cost that may limit its general acceptability, especially when a potential diabetic recipient has little evidence of renal impairment and does not need a kidney transplant.

Within the past 20 years, pancreatic islet transplantation has become a clinical reality and an option in the treatment of IDDM. Islet transplantation has a distinct advantage over whole pancreas transplantation in regards to reduced peri-procedure morbidity. The procedure avoids major surgery and the risk of associated post-operative complications, re-laparotomy and acute (vascularized) graft loss. Islet transplantation, with its ability to be cultured for a period of time prior to transplantation, also offers the future possibility of reducing the immunogenicity (both allo and auto) of the tissue such that little or no immunosuppression will be required.

Early efforts to treat IDDM patients with pancreatic islet transplantation were mostly unsuccessful. Although the first human pancreatic islet allografts were performed in 1974[45], it wasn't until 1991 that a pancreatic islet transplant recipient achieved sustained euglycemia off insulin (for 1 year)[46]. In 2000, the Edmonton group reported 7 consecutive islet transplant recipients achieving insulin independence. All recipients received islets from 2 pancreas donors (one received islets from 4 donors), and were maintained on a glucocorticoid-free immunosuppression protocol using Sirolimus and low-dose Tacrolimus[13]. The success of the Edmonton program has led to a general acceptance that islet transplantation is a clinically feasible therapy, which may be considered for the treatment of patients with IDDM, especially when accompanied by severe hypoglycemia. Since the report of success from Edmonton interest has grown in islet transplantation and now more than 40 centers in North America and many more worldwide are performing this procedure[47].

Patient selection

Pancreatic islet transplant, in general, has been restricted to patients with IDDM who suffer from hypoglycemic unawareness or metabolic instability, or have early evidence of secondary complications due to their diabetes. The patients require long-term, calcineurin-based immunosuppression and thus are subjected to the risks of these agents, such as nephrotoxicity, infection and malignancy. The patient may also become sensitized due to their alloimmune response to the transplant, potentially interfering with subsequent transplantation. Patients with evidence of significant diabetic renal impairment are excluded from islet transplantation until they either require or have undergone a kidney transplant. To improve the likelihood of attaining euglycemia off insulin, most transplant programs and clinical trials will restrict islet transplantation therapy to patients weighting less than 70 kg, a body mass index (BMI) < 27 and not requiring an excessive amount of daily insulin for glycemic control[47-51].

PANCREATIC ISLET TRANSPLANT PROCEDURE

Islet isolation and culture

One of the key elements attributed to the success of the Edmonton program (Edmonton Protocol) was the need to transplant high-quality, purified islets in sufficient numbers. This usually requires isolation of islets from two or more whole pancreata. Refinement of the islet isolation process, with the standardizing of pancreas digestion (the universal use of controlled pancreatic duct perfusion with collagenase and the Ricordi digestion chamber) and use of the COBE cell processor (continuous gradient purification system) for islet purification from exocrine tissue now allows many transplant centers a source of high-quality islets[52-54]. Although some centers transplant the isolated, purified pancreatic islets immediately, other centers maintained the islets in culture for a short period (up to 48 h) prior to transplantation. Holding islets in culture for a short period does not seem to have a detrimental affect on their viability and function and allows the transplant center the option of pre-conditioning the islet tissue (to reduce immunogenicity or improve post-transplant viability), or initiate immunosuppression treatment of the recipient prior to the transplant[55-57].

In the islet recipient, access to the portal vein is obtained by either percutaneous transhepatic portal venous catheterization or mini-laparotomy. The pancreatic islets are suspended in an albumin solution and infused by gravity, along with heparin through the portal vein to embolize in the liver. Prophylactic anticoagulation is continued for several days to reduce the likelihood of an instant blood-mediated inflammatory reaction (IBMIR) with subsequent clot formation and inflammatory

response that has been shown to lead to islet damage[58-60]. Islet recipients are routinely given exogenous insulin to maintain blood glucose levels in a physiologic range as hyperglycemia in the early post-transplant period has been shown to be detrimental to islet function and may interfere with islet engraftment[55,56,61].

Procedural complications

Pancreatic islet transplantation is a less invasive alternative to whole pancreas transplantation and has been shown to be associated with a much lower risk of serious complications. The majority of serious adverse events reported in 2006 by the Collaborative Islet Transplant Registry (CITR) were related to the infusion procedure: bleeding, hematoma and portal vein thrombosis (41.9%)[47]. Newer catheters and radiologic techniques for sealing the intra-hepatic tract used for portal vein infusion of the islets has resulted in a reduction in the incidence of post procedural bleeding[62]. Increased purity of the islet preparation with a subsequent decreased total islet volume infused into the liver may also reduce the likelihood of portal pressure elevation, a risk factor that has been associated with bleeding, in particular, with a second or subsequent islet transplant. The routine use of anticoagulation has likely limited the incidence of portal vein thrombosis, however has been shown to be a factor in the rate of procedural bleeds. Following transplantation, liver transaminases typically are elevated; however routinely return to the normal range within 4 wk[50]. The long-term consequences of intra-hepatic islet infusion are not yet known. The Edmonton group has reported changes consistent with fatty liver in 8 of their first 36 patients on magnetic resonance imaging following transplant.

Immunosuppression

Since 1999, in the US the majority of islet transplant programs (> 90%) use Sirolimus and Tacrolimus in combination as maintenance immunosuppression (Edmonton Protocol). All programs used one or more induction immunosuppression agents at the time of the first islet infusion, the most common being a non-depleting monoclonal anti-CD25 antibody. Complications related to immunosuppression therapy were the second most common severe event reported by CITR (29.6%). Whereas some studies have reported that islet transplant recipients may demonstrate evidence of deterioration of renal function (immunosuppression related) especially where creatinine clearance is already significantly impaired, others have reported no deleterious renal effect following islet transplant[14,47,51].

OUTCOMES OF PANCREATIC ISLET TRANSPLANTATION

Collaborative islet transplant registry

In a survey of all North American transplant programs by CITR, 31 active programs reported 593 islet infusion procedures in 319 recipients during the period 1999-2005[47]. CITR has information on 225 of the 319 allograft recipients (71%) and 425 of the 593 infusion procedures (72%) from 23 participating centers. Sixty-four of the recipients (28.4%) received one islet infusion, 122 (54%) received two, 38 (17%) received three, and one received a total of four islet infusions. Of the 225 recipients, 203 (89%) received an islet transplant alone, while 22 recipients (10%) had previously received a kidney transplant prior to the islet transplant. Insulin independence (off insulin for 14 days or more) was achieved by 69.7% of islet recipients at some point following their last transplant (within two years). Considering all participants, 46.6% remained insulin independent for at least one year following their last transplant and 33.3% were insulin independent at 2 years. Patients who had achieved insulin independence at any point, at one year had a basal C-peptide level (1.1 ng/mL, SD 0.65), and hemoglobin A1c (6%, SD 0.8) in the normal (or near normal) range. Severe hypoglycemic events were shown to be dramatically deceased following the first islet infusion. Greater than 85% of recipients had one or more severe hypoglycemic event prior to transplant, compared to less than 5% in the first year following the transplant.

International trial of the edmonton protocol for islet transplantation

In 2006, a study organized by the Immune Tolerance Network (initiated by the National Institutes of Health) among six North American and three European transplant centers to assess the feasibility and reproducibility of the Edmonton Protocol reported their results[14]. Each center was confined to use the islet isolation technique and immunosuppression protocol as described by the Edmonton group. In the period 2001-2003, 36 patients underwent 77 islet infusion procedures. Eleven of the recipients (31%) received one islet infusion, 9 (25%) received two and 16 (44%) received three infusions. Insulin independence was achieved by 21 patients (58%) at some point, 16 patients (44%) remained insulin independent for one year and 5 (14%) remained insulin-independent at 2 years following their last transplant (35 patients evaluated at 2 years). Although most patients had returned to requiring insulin by two years, C-peptide remained detectable in 70%, demonstrating persistence of islet function. Average hemoglobin A1c levels were in shown to be in the normal range for patients who remained insulin-independent at 2 years, and only slightly elevated above normal when partial graft function was demonstrated. All patients with residual islet function were completely protected from severe hypoglycemic episodes.

Secondary complications of IDDM

Successful islet transplantation, with correction of glycemic control has only been a clinical reality for a short period of time and there has been little published to-date on the effect of islet transplantation on the secondary complications (microvascular, neurologic and macrovascular diseases) associated with IDDM. Assessment of the long-term safety and effectiveness of islet transplantation for the treatment of IDDM will require the future report of more centers with longer periods of follow-up.

CONCLUSION

Both whole pancreas transplantation and pancreatic islet transplantation have been shown to be successful at creating a euglycemic state in a patient with IDDM with preprandial and postprandial blood glucose concentrations and hemoglobin A1c levels comparable to those in the non-diabetic population. Both forms of transplants are also effective at eliminating the occurrence of significant hypoglycemic events (even with only partial islet function evident). Whole pancreas transplantation has also been shown to be effective at maintaining a euglycemic state over a sustained period of time, and thus provides an opportunity for a recipient to benefit from improvement of their blood glucose control (halt or reverse secondary complications of IDDM). Islet transplantation is attractive as a less invasive alternative to whole pancreas transplantation and offers the future promise of immunosuppression-free transplantation through pre-transplant culture. Although recent advances have led to an increased rate of obtaining insulin-independence following islet transplantation, further developments are needed to improve the long-term viability and function of the graft to maintain improved glucose control over time.

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LIVER CANCER

Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study

EM Jung, DA Clevert, AG Schreyer, S Schmitt, J Rennert, R Kubale, S Feuerbach, F Jung

EM Jung, AG Schreyer, J Rennert, Feuerbach S, Department of Diagnostic Radiology, University Hospital Regensburg, Franz-Josef Strauss-Allee 11, Regensburg 93043, Germany
DA Clevert, Department of Radiology, Ultrasound Section, Großhadern Hospital, Ludwig Maximilian University of Munich, Marchioninistr, 15, München 81377, Germany
S Schmitt, Department of Trauma Surgery, Passau Hospital, Innstraße 76, Passau 94034, Germany
R Kubale, Institutes of Radiology, Nuclear Medicine and Ultrasound, Pirmasens Hospital, Germany
F Jung, Center for Biomaterial Development, Institute of Polymer Research, GKSS, Kantstr, 55, Teltow 14513, Germany
Correspondence to: Ernst Michael Jung, MD, Department of Radiology, University of Regensburg, Franz-Josef Strauss-Allee 11, Regensburg 93053, Germany. emjung@gmx.de
Telephone: +49-941-9447401 Fax: +49-941-9447402
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Abstract

AIM: To establish the extent to which contrast enhancement with SonoVue in combination with quantitative evaluation of contrast-medium dynamics facilitates the detection of hepatic tumors.

METHODS: One hundred patients with histologically confirmed malignant or benign hepatic tumor (maximum size 5 cm) were analyzed. Contrast-enhanced ultrasound (bolus injection 2.5 mL SonoVue) was carried out with intermittent breath-holding technique using a multifrequency transducer (2.5-4 MHz). Native vascularization was analyzed with power Doppler. The contrast-enhanced dynamic ultrasound investigation was carried out with contrast harmonic imaging in true detection mode during the arterial, portal venous and late phases. Mechanical index was set at 0.15. Perfusion analysis was performed by post-processing of the raw data [time intensity curve (TIC) analysis]. The cut-off of the gray value differences between tumor and normal liver tissue was established using Receiver Operating Characteristic (ROC) analysis 64-line multi-slice computed tomography served as reference method in all cases. Magnetic resonance tomography was used additionally in 19 cases.

RESULTS: One hundred patients with 59 malignant (43 colon, 5 breast, 2 endocrine metastases, 7 hepatocellular carcinomas and 2 kidney cancers) and 41 benign (15 hemangiomas, 7 focal nodular hyperplasias, 5 complicated cysts, 2 abscesses and 12 circumscribed fatty changes) tumors were included. The late venous phase proved to be the most sensitive for classification of the tumor type. Fifty-eight of the 59 malignant tumors were classified as true positive, and one as false negative. This resulted in a sensitivity of 98.3%. Of the 41 benign tumors, 37 were classified as true negative and 4 as false negative, which corresponds to a specificity of 90.2%. Altogether, 95.0% of the diagnoses were classified as correct on the basis of the histological classification. No investigator-dependency (P = 0.23) was noted.

CONCLUSION: The results show the possibility of accurate prediction of malignancy of hepatic tumors with a positive prognostic value of 93.5% using advanced contrast-enhanced ultrasound. Contrast enhancement with SonoVue in combination with quantitative evaluation of contrast-medium dynamics is a valuable tool to discriminate hepatic tumors.

Key words: Liver tumors; Malignant tumor; Contrast harmonic imaging; Quantitative contrast enhancement

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INTRODUCTION

Benign liver lesions have a prevalence of approximately 20% in the whole population, whereas tumor patients show hepatic metastases in 25%-50% at the time of diagnosis. The liver is the main region for the manifestation of distant metastases in oncological diseases. Therefore, reliable detection of hepatic metastases is a prerequisite for evaluating the prognosis and establishing a therapeutic procedure. To evaluate the prospects of resection or to plan interventional therapy, it is essential to establish the number and location of the lesions. Prior to this, the focal hepatic lesion must be accurately characterized. Since no

single diagnostic tool has been able to clarify this question with sufficient reliability up to now, histological analysis remains the gold standard. Characterization of focal hepatic lesions with imaging techniques is an important part of radiological diagnosis and is still the focus of scientific research because of technical innovations of existing methods[1-15]. Special attention has been paid to the validation of new methods, especially the further development of contrast-enhanced ultrasound techniques because ultrasound is the most common and best available imaging technique[1-3,5-14].

In a patient with a known primary malignancy, any focal liver lesion seen on non-enhanced ultrasound must be regarded as suspicious of metastasis. However many lesions (25%-50% of lesions ≤ 2 cm) will eventually prove to be benign; next to contrast-enhanced ultrasound, other imaging modalities or biopsies are used to further characterize the lesion. For lesions < 1 cm, the false-negative rate of non-enhanced ultrasound is as high as 80%. Therefore contrast-enhanced ultrasound should be carried out in addition to conventional ultrasound in most cancer patients for definitive liver staging[6-9].

This prospective two-center study investigated the possibility of evaluating the malignancy of liver lesions on the basis of contrast-enhanced harmonic imaging (CHI) and SonoVue, by comparing the dynamics of the contrast agent in tumor lesions with those in the surrounding tissue. The objective of this study was to examine to what extent the detection of liver tumors will be simplified after contrast application with SonoVue, in combination with quantitative measurement of contrast agent dynamics. Special attention was paid to the feasibility of assessing the malignancy of the tumor by quantitative measurement of the tumor tissue contrast compared to the surrounding healthy tissue.

MATERIALS AND METHODS

Study design

This prospective study was performed at two centers. The equipment (Logic 9; GE Healthcare, Milwaukee, WI, USA), transducers (4C probe), data acquisition (PACS connection) and display, as well as the procedure of ultrasound examination were set identically at both centers.

Only patients with histologically confirmed malignant or benign hepatic lesions were included in the study. Tumor sizes were differentiated as follows: < 3 cm, and 3-5 cm. The maximum number of lesions considered was five.

Exclusion criteria were: tumor lesion > 5 cm, and more than five lesions, strong allergic reactions, diseases of liver and kidney with confirmed elevation of laboratory parameters, acute heart failure, acute myocardial infarction, subcutaneous emphysema, meteorism, tachypnea, and aerobilia.

Operational sequence and follow-up

A biopsy was taken from the malignant liver tumors to establish the histological result (if necessary, surgical resection or radiofrequency ablation was performed). In case of hemangioma, constant results of ultrasound examinations over 2 years and additional MRI or multiphase CT with constant results over 2 years was required. All contrast-enhanced ultrasound investigations were conducted with a multi-frequency linear transducer (2.5-4 MHz, Logic 9; GE Healthcare). Transmitted energy was reduced to a magnitude of < 30% with a mechanical index (MI) of 0.15. In fundamental B-scan, an optimal depth adjustment with three focus zones was made. After B-scan and analysis of vascularization with power Doppler ultrasound, an intravenous bolus injection of 2.5 mL SonoVue (Altana, Bracco, Konstanz, Germany) was administered through a 20-18 Ga peripheral cubital cannula, followed by a bolus injection of 10 mL NaCl.

Using CHI, the microbubbles of the contrast medium were stimulated to vibrate and their energetic harmonic Doppler frequencies were employed for imaging. In the technique of pulse inversion harmonic imaging (PIHI) of CHI, emission frequencies are digitally encoded. Several pulses, each after a defined period of time, were produced. Then the received echos were subtracted from each other. The harmonic frequencies of contrast-enhanced echos in the flowing blood remained as image information. In the subtraction mode of the background information, the influx of contrast agent and its distribution were imaged, even with very low acoustic energy.

A low MI allowed the real-time evaluation of the contrast-agent enhancement. CHI suppressed the fundamental linear echoes from the liver tissue, whereas the non-linear echoes reflected from the microbubbles remained, which provided the ultrasound signal. The true agent detection mode was also used to process the fundamental non-linear signal generated by ultrasound contrast agents that were stronger than the harmonic signal, thereby increasing the specificity of the microbubble-to-tissue ratio. The combination of SonoVue with true agent detection mode using a low MI allowed dynamic enhancement of the blood supply of a liver nodule to be evaluated during the various phases of contrast-agent circulation. Perfusion curves described the ultrasound signal intensity over time after contrast bolus injection in a region of interest (ROI).

Modified ultrasound slices close to the tumor enabled a minimum depth of penetration to be maintained. One turn of the scanner head examined the whole liver during intermittent breath holding. Digital data from cine sequences allowed post-processing of the dynamic tumor blood flow and calculation of 3D data block images. Scanning was carried out during the arterial (< 30 s), portal venous (40-120 s) and late venous (> 120 s) phases in true agent detection mode. Parallel dynamic imaging in fundamental B-scan and recording of the dynamics of contrast agent in the subtraction mode of CHI were performed in the meantime, which greatly facilitated the localization of tumor lesions.

Color-coded Doppler sonography and power Doppler ultrasound were used to evaluate native vascularization. Color enhancement was adjusted to the lowest possible pulse repetition frequency (PRF, < 1000 Hz) and to the best possible, artifact-free color enhancement, in order to avoid artifacts. The complete data of the contrast-agent examination were recorded in up to 5 min. The length of

the specific cine loops in the three stages was a minimum of 20 s each.

Measurements were done in the arterial phase (to quantify arterial vascularization), in the portal venous phase (to asses contrast-agent accumulation, pooling), and up to 5 min in the late phase (to assess the wash-out effect).

Altogether, five measurements were performed in each patient in the ROIs: one measurement within the lesion (T1) and four in the surrounding liver parenchyma at 12, 3, 6 and 9 o'clock positions (G1, G2, G3 and G4), with ROIs not larger than 5 mm. A careful adjustment of the position of the ROIs was effected manually depending on inspiration or expiration. Time intensity curve (TIC) analyses were made off-line and then transferred into an spreadsheet table (Excel 2003; Microsoft, Redmond, WA, USA) for off-line analysis. In addition, digital raw data were recorded and saved.

The different dynamics of the depletion of contrast agent - in comparison to the depletion within normal liver tissue - was used to assess the malignancy of the tumors. For this purpose, the recorded gray scale parameters of the ROIs were averaged over the time of recording, and then the following gray scale differences were calculated:

arterial phase: Diffart = T1art - (G1art + G2art + G3art + G4art)/4

portal venous phase: Diffpv = T1pv - (G1pv + G2pv + G3pv + G4pv)/4

late venous phase: Difflv = T1sv - (G1sv + G2sv + G3sv + G4sv)/4

The malignancy of the tumor was then inferred from the absolute value of the differences. If the absolute value of Diffsv was smaller than -0.4 in the case of a malignant tumor, the assessment was rated as true positive, if the value was greater than -0.4, the assessment was rated as false negative. If the value was greater than -0.4 in the case of a benign tumor, the assessment was rated as true negative, otherwise as false positive. First, the critical value had been ascertained in a small preliminary study, which had shown characteristic contrast-agent dynamics in malignant liver tumors. The determination of the optimal scale value is shown in the section ROC analysis. Figure 1A-C presents an example of contrast-agent dynamics.

An increased wash-out of contrast agent molecules appeared to occur in the malignant tumor vessels rather than in normal hepatic vessels. This was probably caused by arteriovenous shunts within the lesion and additional tumor necrosis, which prevented accumulation of microbubbles in the malignant tumor. Therefore, the tumor was more hypoechoic, especially in the late phase, i.e., the gray value imaging was less distinct. This characteristic behavior could then be used for quantitative evaluation of gray value differences in the diagnosis of liver tumor malignancy.

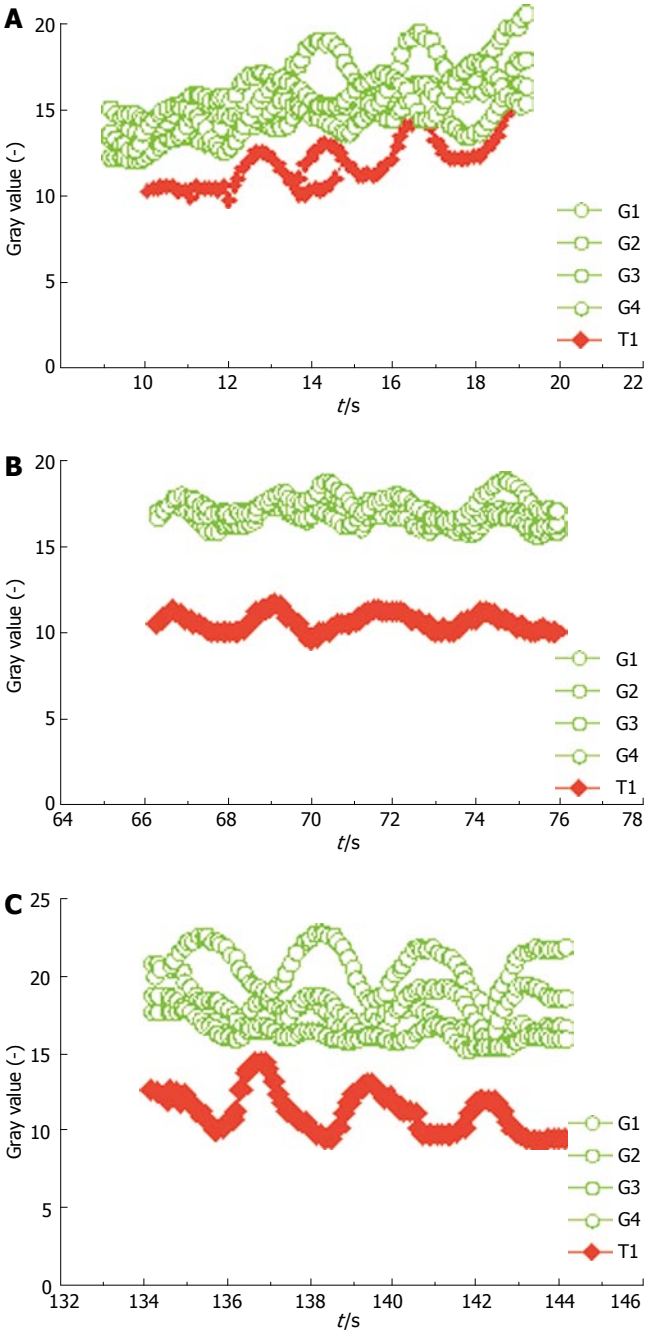

Figure 1 Gray value progression of the five ROIs over 10 s. **A**: For a patient with liver metastasis in the arterial phase; **B**: For the same patient in the portal-venous phase; **C**: For the same patient in the late venous phase. (red, gray-value progression in the tumor; green, gray-value progression in the surrounding healthy tissue).

Ethical concerns

The study data were collected within the framework of an external quality control as a registry complying with the principles of the Helsinki/Edinburgh Declaration of 2002. Before each contrast agent application, patients were informed in detail about possible risks such as allergic reactions. The consent of all patients was obtained prior to the study.

Statistical analysis

All results were presented as the mean ± SD. Specificity, sensitivity, positive and negative prognostic value, as well as diagnostic accuracy were calculated to evaluate their diagnostic significance. Since there were, as a rule, no clinical findings or the history of the patient was not available, the examiner could not establish a pretest probability. In this situation, the resulting sensitivity and specificity of the three diagnostic tests were calculated on

Table 1 Results based on contrast-enhanced CHI during the arterial phase

	Tumor present	Tumor not present	
Test positive	40	19	59
Test negative	19	22	41
	59	41	100

Table 2 Results based on contrast-enhanced CHI during the portal-venous phase

	Tumor present	Tumor not present	
Test positive	54	10	64
Test negative	5	31	36
	59	41	100

the basis of the following table[17]:

	Disease existent	Disease non-existent	
Test positive	a	b	a + b
Test negative	c	d	c + d
	a + c	b + d	

Where a is the number of patients with existing disease and a positive test result (true positive); b is the number of patients with no disease, but a positive test result (false positive); c is the number of patients with existing disease and a negative test result (false negative); d is the number of patients with no disease and a negative test result (true negative); a/(a + c): sensitivity: true positive results/ number of affected patients; d/(b + d): specificity: true negative results / number of healthy patients.

Using the Bayes formula it was possible to calculate the probability[17] that disease was present, on the basis of a positive result of the diagnostic test (positive prognostic value: pV).

$$pV = \frac{\text{true positive results}}{(\text{true positive results} + \text{false positive results})}$$

It was also possible to calculate the probability of no disease on the basis of a negative test result (negative prognostic value: nV).

$$pV = \frac{\text{true negative results}}{(\text{true negative results} + \text{false negative results})}$$

RESULTS

Patient characteristics

Altogether, 100 consecutive patients (43 women, 57 men, aged 25-83 years; mean 57 years) with suspicion of hepatic tumor were included in the study. Fifty-nine of these suffered from a malignant tumor (43 metastases of colon carcinoma, 5 metastases of breast cancer, 2 endocrine metastases, 7 HCC metastases, and 2 renal cell carcinoma), 41 patients had a benign tumor (9 hemangioma, 6 high-flow hemangioma, 7 nodular hyperplasia, 5 complicated cysts, 2 abscess, and 12 circumscribed fatty degeneration of the liver (focal hyposteatosis) with constant results over a minimum of 2 years. Maximum tumor size was 5 cm.

Table 3 Results based on contrast-enhanced CHI during the late phase

	Tumor present	Tumor not present	
Test positive	58	4	62
Test negative	1	37	38
	59	41	100

Table 4 Diagnostic value of quantitative CHI for detection of malignant hepatic tumors in different phases after intravenous application of ultrasound contrast agent

	Arterial phase	Portal-venous phase	Late venous phase
Specificity (%)	53.7	75.6	90.2
Sensitivity (%)	67.8	91.5	98.3
Positive prognostic value	67.8	84.4	93.5
Negative prognostic value	53.7	86.1	97.4

Analysis of contrast-agent dynamics

Arterial phase: Of the 59 patients with histologically confirmed malignant tumors, 40 were classified as true positive and 19 as false negative in the arterial phase. Of the 41 patients with histologically classified benign tumor, 22 were classified as true negative and 19 as false positive (Table 1, Figure 1A). In the arterial phase, a sensitivity of 67.8% with a specificity of 53.7 % (pV 67.8%, nV 53.7%) was achieved. Therefore, 62% of the test results were correct.

Portal venous phase: Of the 59 patients with histologically confirmed malignant tumor, 54 were classified as true positive and 5 as false negative in the portal venous phase. Of the 41 patients with histologically classified benign tumor, 31 were classified as true negative and 10 as false positive (Table 2, Figure 1B). This resulted in a sensitivity of 91.5% with a specificity of 75.6%, which gave a pV of 84.4% and nV of 86.1%. The correctly interpreted test evidence therefore amounted to 85.0%.

Late venous phase: Of 59 patients with histologically confirmed malignant tumor, 58 were classified as true positive and 1 as false negative in the late phase. Of 41 patients with histologically confirmed benign tumor, 37 were classified as true negative and 4 as false positive (Table 3, Figure 1C). This resulted in a sensitivity of 98.3% with a specificity of 90.2%, which gave a pV of 93.5% and nV of 97.4%. In the late venous phase, the correctly interpreted test evidence amounted to 95.0%.

A comparison of the diagnostic values of the three phases shows that the best accuracy was achieved in the late venous phase (Table 4).

Stability of diagnostic criteria

The sensitivity over the course of the study provided a sensitive indicator of the diagnostic value of this method in identifying malignant liver tumors in 100 patients (Figure 2). Only the late venous phase was analyzed since

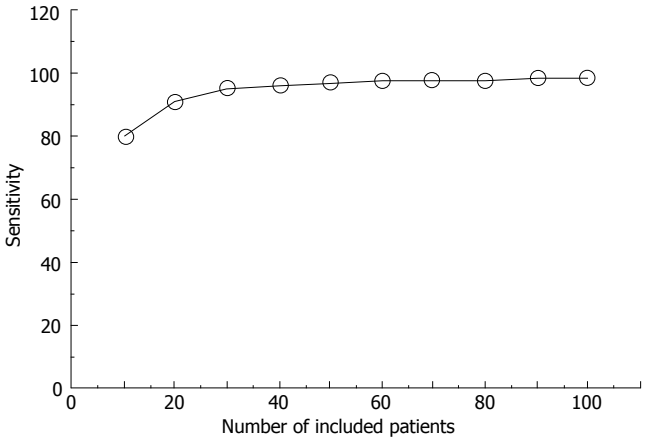

Figure 2 Sensitivity of the diagnostic test in relation to the number of patients examined. The sensitivity over the course of the study provides a sensitive indicator to the diagnostic value of this method in identifying malignant liver tumors in 100 patients.

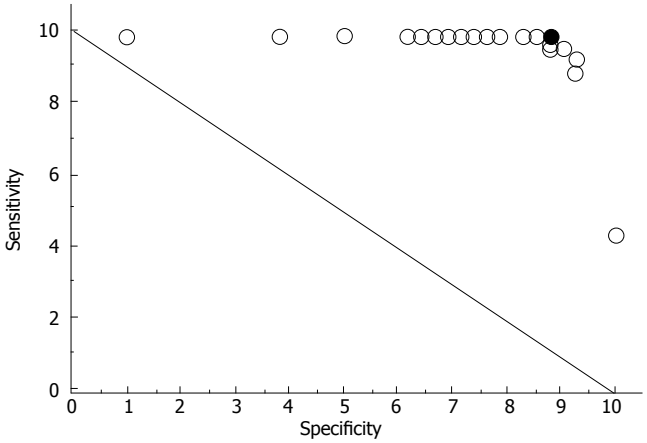

Figure 3 ROC diagram showing the specificity and sensitivity in the late venous phase for different limiting values (varying the cut-off values from +5 to -5). A greater distance from the diagonal line indicates that a diagnostic test had a higher reliability. The black point in the ROC diagram corresponds to the best sensitivity and specificity.

it showed the most sensitive results. Sensitivity over the course of the trial was cumulatively calculated after every 10 patients. This showed that sensitivity very clearly approached a stable final value. This meant that sensitivity would not have changed significantly if more patients were included. Therefore, a valid evaluation could be made. The other diagnostic parameters showed similar results.

ROC analysis

The test results depended on the cut-off value of the gray value differences that were chosen. By modifying the cut-off value by means of an ROC analysis, it was possible to attain the optimum selectivity by improving sensitivity and specificity[18]. Figure 5 shows the respective specificity and sensitivity for the late venous phase for different limiting values (varying the cut-off values from +5 to -5). Usually, the greater the distance from the points to the diagonal, the better the diagnostic test. It is evident that the point marked in red in the ROC diagram was the one with

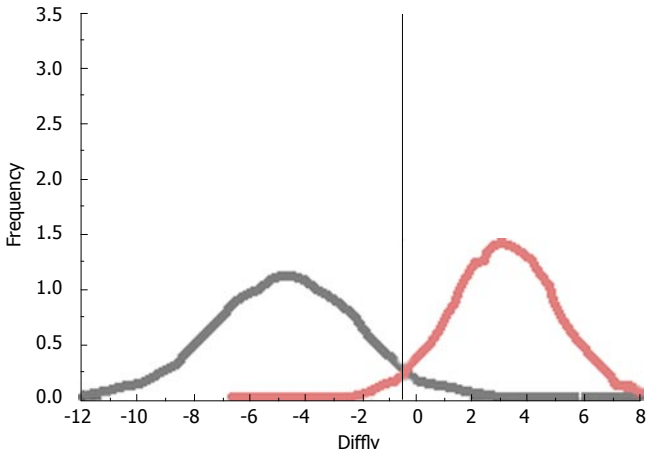

Figure 4 Distribution of the characteristic gray-value differences in the late venous phase for patients with benign (black) or malignant (red) hepatic tumor.

Table 5 Specificity and sensitivity for both testing centers

	Center 1 *n* = 50	Center 2 *n* = 50	Total *n* = 100
Specificity (%)	85.7	95.0	90.2
Sensitivity (%)	100	96.7	98.3
Negative prognostic value	100	95.0	97.4
Positive prognostic value	90.6	96.7	93.5

the most sensitivity and specificity. This optimum cut-off value was easy to calculate [cut-off valueopt = 0.5 × (specificity + sensitivity)/2] (Figure 3). The optimal cut-off value between the two groups of patients was readily established. The best selectivity for the two groups was at a cut-off value of -0.4 (Figure 4).

Center dependence

The diagnostic parameters were calculated and compared for the two centers (Table 5). Variations in the parameters were based on one single case in the whole study with a false-positive value in only one center, and a single case was still too small for a separate description. The quality of testing did not differ between the two centers: 47 out of 50 patients were classified correctly in center 1, and 48 out of 50 in center 2. There was therefore no center-dependence ($P = 0.229$) (Figure 5A-F).

DISCUSSION

All patients with suspected hepatic lesions, who were sent for further diagnostic workup to the two different centers, were consecutively included in the study. First, an ultrasound examination of the liver was undertaken using B-scan with a high-resolution multifrequency probe, resulting in a complete digital data set of the whole liver. Then, vascular ultrasound with power Doppler was performed to assess the vascularization of tumor lesions detected by the B-scan. After a bolus injection of 2.5 mL SonoVue, quantitative dynamic contrast-enhanced ultrasound was performed in low MI Technique for the arterial, portal-venous and late venous phase. As for

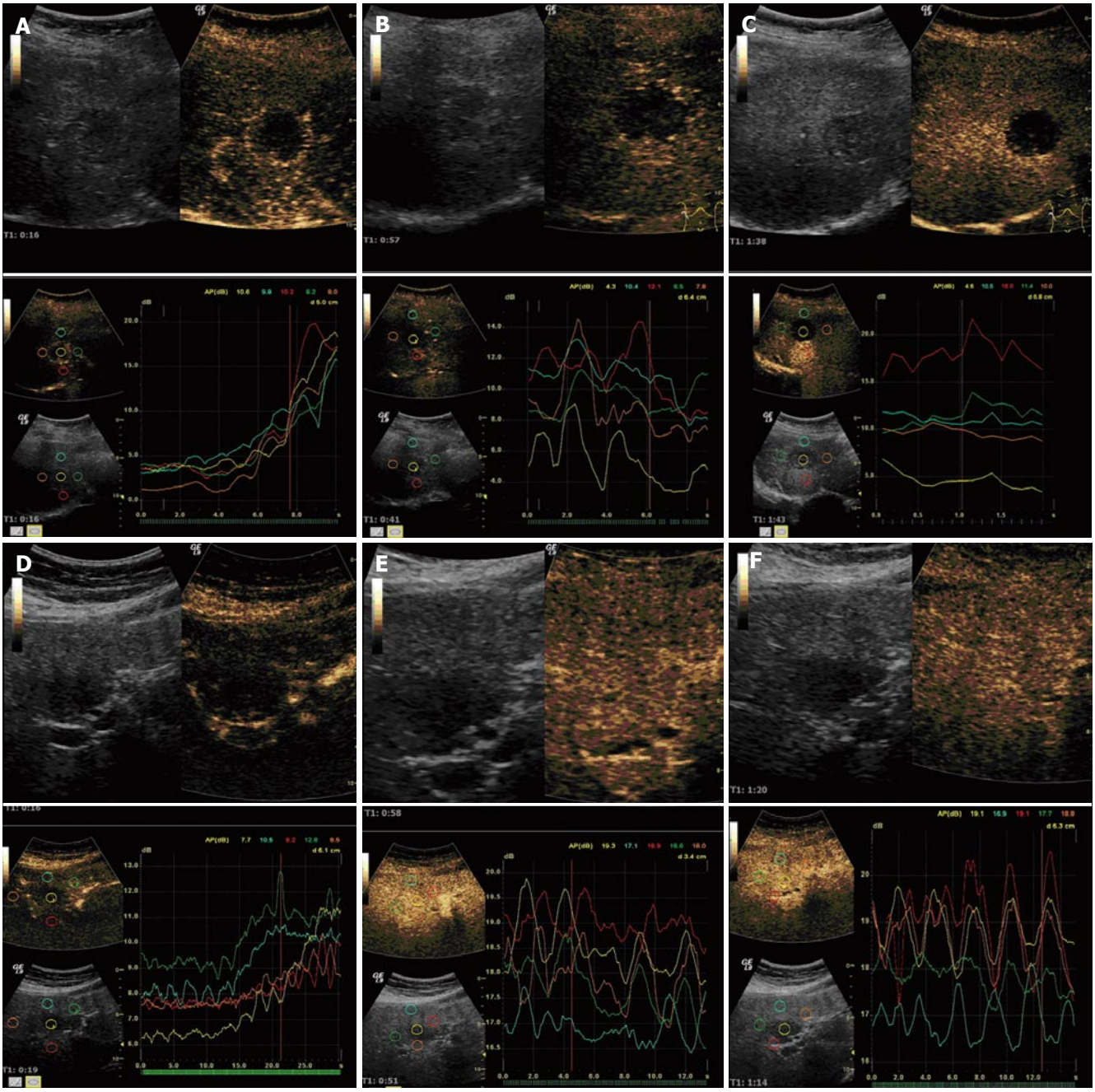

Figure 5 True agent detection mode of CHI with TIC analysis. **A**: Malignant lesion in the arterial phase. Arterial enhancement of the tumor margin - metastasis of beast cancer; **B**: Malignant lesion in the portal-venous phase. Lower enhancement of the tumor - metastasis of beast cancer; **C**: Malignant lesion in the late venous phase (> 100 s). Lower enhancement of the tumor - metastasis of beast cancer; **D**: Benign lesion in the arterial phase. Lower enhancement of the tumor-adenoma histological confirmed in the early arterial phase; **E**: Benign lesion in the portal-venous phase. Enhancement of the tumor - adenoma histological confirmed-similar to normal liver tissue; **F**: Benign lesion in the late venous phase. Enhancement of the tumor-adenoma histological confirmed - similar to normal liver tissue.

the Contrast Harmonic Imaging (CHI) technique, Pulse Inversion Harmonic Imaging (PIHI) with simultaneous data acquisition of the B-image and the contrast-enhanced perfusion image in True Agent Detection mode was used. Data were stored with dynamic and cine sequences up to a maximum of 60 s. Contrast -agent dynamics were represented in terms of the digital raw data of the gray values, exported into a spreadsheet table, and evaluated by an external institution without any knowledge of imaging and the clinical data. The histological result was only communicated after the end of the study, so that diagnostic criteria were analyzed without any previous knowledge. An information bias was therefore precluded (which is especially important for radiological imaging), which enabled the prognostic values to be calculated according to Bayes[18]. Diagnostic tests were therefore evaluated in the same way as in the specific clinical situation.

Conventional ultrasound methods are limited for depicting and characterizing focal liver lesions by a low contrast between the lesion and normal parenchyma. The use of specific contrast agents for ultrasound improves the diagnostic value of conventional ultrasound and enables a complete diagnosis of liver lesions. Solutions for a complete ultrasound diagnosis have been proposed

with the introduction of second-generation contrast agents and the use of low MI techniques, such as pulse inversion, which allows a continuous sweep with the same bubbles[1-3,5-10]. The support of gray-scale images is essential to delineate the lesions better and to check the perfect matching of contrast-enhanced and B-mode images. The availability of the true agent detection mode is able to overcome these limitations by higher signal sensitivity and simultaneous acquisition of gray scale and contrast-enhanced images.

SonoVue provides strong and persistent harmonic resonance at low MI ($\leqslant$ 0.2), with which minimal or no bubble destruction occurs. This allows for continuous real-time imaging of a lesion during its vascular phase. With real-time low-MI imaging, the dynamic enhancement pattern and the vascular morphology of a lesion is assessed during the arterial phase (10-20 s until 25-50 s after bolus injection) and portal-venous phase (30-45 s until 120 s after bolus injection). The delayed phase (> 120 s after bolus injection) is particularly useful for the detection of, as they show as non-enhancing defects. Characterization is also improved by the late phase, as the vast majority of benign lesions show contrast uptake in this phase[5,7,8]. Our results show that by means of CHI and subsequent quantitative gray-value analysis of contrast-agent dynamics in the late venous phase, it is possible to assess malignant liver tumors with a sensitivity of 98.3% with an nV of 97.4%.

High-resolution digital contrast-enhanced ultrasound techniques have considerably improved the assessment of malignant and benign tumor lesions in the liver[1-12]. Under optimal examination conditions that enable imaging of the entire liver, it is possible to attain a diagnostic reliability comparable to that of contrast-enhanced MRI with a liver-specific contrast agent, and an even higher diagnostic certainty of > 90%, compared with contrast-enhanced multi-detector-spiral CT[1,4,15]. Using ultrasound contrast agents, a signal amplification of up to 10 dB is feasible. This allows a better differentiation between regular liver tissue and malignant tissue within the liver, which has an increased contrast wash-out in the portal-venous and, especially, in the late phase. The perfusion curves in healthy liver tissue show a slow increase, which reaches a plateau at the portal-venous phase, followed by a very slow decrease. In most benign tumors, enhancement can also be detected in the late phase after contrast agent injection. In cases of malignancy, the decrease begins in the portal-venous phase in most cases after slow marginal arterial enhancement. Tumor lesions of HCC, liver adenoma, high-flow hemangioma and focal nodular hyperplasia can show an early arterial enhancement in the first 30 s in perfusion curves[4,6,8,12,13]. These lesions may be masked in the portal-venous phase. Therefore, the most important phase for tissue differentiation is the late phase, because malignant tissue shows a contrast-agent wash-out in the late phase, whereas regular tissue still has a slowly descending plateau. This is the explanation for the significant differences of the gray-value analysis of the contrast-enhanced TIC analysis in the late phase.

Using a continuously acquired contrast-enhanced dynamic CHI, the diagnostic accuracy in characterization of benign lesions, such as partially thrombosed or high flow hemangioma, focal nodular hyperplasia or local fatty and regenerative changes, is superior to that of B-scan or contrast-enhanced spiral CT[1,2,5,8,10].

The general problem for liver ultrasound still remains unchanged. The subdiaphragmatic liver segments Ⅳa and Ⅷ are difficult to visualize, which makes it very difficult to assess a lesion within these segments. Investigation of only part of the liver was possible owing to the high attenuation of ultrasound especially in the cranial and dorsal parts of the liver where the position of the liver was very high under the diaphragm or when patients had difficulty holding their breath. Secondly, fatty areas or cirrhosis of the liver, as well as lesions location deeper than 10 cm, diminish the penetration of contrast-specific imaging modes, which results in a decreased signal noise[1,5,8-10]. Another major problem for all imaging modalities such as contrast-enhanced CT or MRI remains the detection and characterization of lesions smaller than 5 mm[7].

SonoVue is a pure intravascular blood-pool contrast agent without a specific liver phase. The microbubbles help to visualize small parenchymal vessels. Malignant liver lesions such as metastases or HCC lesions are characterized by demarcation during the late phase. SonoVue late phase is based on visualization of parenchymal vessels. It is possible for very small HCC lesions, hemangiona or adenoma with vascular architecture comparable to that of the liver parenchyma to be missed in the detection study. Hypervascularization is a feature of HCC with a diameter > 2 cm. This may explain why the early phase of enhancement might not be effective for some very small HCC lesions. Early arterial enhancement can be found in 76%-96% of HCC lesions, and homogeneous enhancement in the late phase in 3%-30% of the patients[4,6,8,12,13].

The detection rate still remains examiner-dependent, even with low-MI contrast-agent techniques. In our study, we were able to demonstrate the feasibility of objective digital raw data analysis, which was not influenced by different examiners in our two-center approach. Therefore, dedicated software for computer-aided diagnosis can be developed, which allows the user an objective assessment of benign or malignant liver lesions based on digital raw data.

Contrast-enhanced ultrasound (CEUS) reveals typical patterns of contrast enhancement in the different lesion histotypes, and provides equivalent accuracy to CT and MRI in focal liver lesion characterization. CEUS should be considered in every patient with a known malignancy with proven or suspected liver metastases at baseline ultrasound during preoperative staging or postoperative follow-up.

Without dynamic evaluation of contrast enhancement, in approximately 10% of cases focal liver lesions remain indeterminate even after microbubble injection, and therefore Gadobenate-Dimeglumine (Gd-BOPTA) enhanced MRI should be employed, followed when necessary by ultrasound-guided biopsy. Standard cross-sectional imaging procedures including multislice CT or Gd-BOPTA-enhanced MRI should be referenced in every case not explorable by ultrasound, or in patients with no evidence of liver metastases at CEUS and with clinically

suspected liver metastases, such as in cases with increased serum levels of tumor markers.

Using dynamic CHI, the malignancy of hepatic tumors can be predicted with a positive prognostic value of 93.5%. CHI with SonoVue in combination with dynamic quantitative evaluation of contrast-agent dynamics is a valuable tool for discrimination.

COMMENTS

Background

Liver lesions are a common diagnostic problem in medical imaging. Contrast-enhanced ultrasound is a recently introduced new modality in lesion assessment. In our study the quantitative evaluation of raw ultrasound data was assessed compared with histology as a gold standard.

Innovations and breakthroughs

Based on this raw data evaluation, new computer-assisted algorithms can be created, which allow automated liver lesion diagnosis.

Applications

For contrast-enhanced ultrasound-based lesion evaluation, three phases (arterial, portal-venous, and late phase) are mandatory. Computer-based dynamic analysis of raw digital ultrasound data facilitates lesion characterization.

Peer review

In this study, the authors review their experience of CHI and its diagnostic value in patients with space-occupying liver lesions. They conclude that using dynamic CHI, the malignancy of hepatic tumors can be predicted with a pV of 93.5%. CHI with SonoVue in combination with dynamic quantitative evaluation of contrast-agent dynamics is a valuable tool for discrimination.

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Analysis of the expression of coxsackievirus and adenovirus receptor in five colon cancer cell lines

Yassan Abdolazimi, Majid Mojarrad, Mehrdad Pedram, Mohammad Hossein Modarressi

Yassan Abdolazimi, Majid Mojarrad, Mehrdad Pedram, Mohammad Hossein Modarressi, Department of Medical Genetics, Medical Sciences/University of Tehran, Tehran, Iran
Mohammad Hossein Modarressi, Reproductive Biotechnology Research Center, Avesina Research Institute, Tehran, Iran
Supported by the Funds from Medical Sciences/University of Tehran and Iranian Ministry of Health and Medical Education
Correspondence to: Dr. Mohammad Hossein Modarressi, Department of Medical Genetics, Medical Sciences/University of Tehran, PO Box 14155-6447, Tehran, Iran. modaresi@tums.ac.ir
Telephone: +98-21-88953005 Fax: +98-21-66404577
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Abstract

AIM: To investigate the expression of coxsackievirus and adenovirus receptor (CAR) and adenovirus-mediated reporter gene transfer in five human colon cancer cell lines.

METHODS: Expression of CAR-specific mRNA and protein was analyzed by reverse transcriptase polymerase chain reaction and Western blotting, respectively. Adenovirus-based gene delivery was evaluated by infection of cells with adenoviral vector carrying the green fluorescent protein (GFP) gene.

RESULTS: All the colon cancer cell lines examined (HT29, LS180, SW480, SW948 and SW1116) expressed CAR full-length mRNA and an alternatively-spliced variant that lacks the transmembrane coding exon. All cell lines were detected as CAR-positive by Western blot analysis. Further, all cells we examined were efficiently infected with adenoviral vector-GFP.

CONCLUSION: The data indicated that the five colon cancer cell lines tested expressed adenovirus primary receptor and could be efficiently infected by adenoviral vectors. Therefore, these cell lines will be useful for adenovirus-based gene transfer and research.

Key words: Coxsackievirus and adenovirus receptor; Adenoviral infection; Gene therapy

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INTRODUCTION

Colorectal cancer is the third most common cancer and among the three top fatal malignancies. In 2007, there were an estimated 153760 new cases and 52180 deaths from colorectal cancer, which corresponds to approximately 10% of all newly diagnosed cancers in the United States[1,2]. Gene therapy has been considered a potential innovative approach for the treatment of colorectal cancer[3,4]. To date, over 600 gene therapy clinical trials have been initiated in the US, 60% of which are cancer-related[5]. Currently, adenoviral vectors are the most frequently administered vectors[6]. This is because of their advantageous features such as a broad range of cell targets, the ability to be produced in high titers, and their accommodation of relatively large segments of DNA[7,8].

Adenovirus initiates infection as well as does adenovector-mediated gene transfer by attachment of the fiber knob to a cell surface receptor, the coxsackievirus and adenovirus receptor (CAR)[9]. CAR, the primary high-affinity receptor for adenovirus, is a 46-ku transmembrane glycoprotein and belongs to the immunoglobulin superfamily[10,11]. The human CAR gene, CXADR, encodes a 365-amino acid protein, and four pseudogenes have been reported on chromosomes 15, 18 and 21 (two copies)[12]. Alternatively spliced variants of CAR have also been identified[13]. Thoelen et al has described three splice variants (β-, δ- and δ-transcripts) of the CAR gene in addition to the normal α-transcript.

Expression of CAR has been studied in numerous cell lines including those of head and neck carcinoma[14], renal cell carcinoma[15], bladder cancer[16], ovarian cancer[17,18], cervical cancer[19], lung and pancreatic cancer[20], prostate cancer[21] and glioma[22]. In all these studies, high expression of CAR correlated with increased adenoviral infection efficiency; cells lacking or expressing low levels of CAR were resistant to adenovirus infection.

Since CAR expression has not been studied in colon cancer cell lines, we examined the expression of CAR at the mRNA and protein levels, along with its splice variants in five human colon cancer cell lines. Adenovirus-mediated reporter gene transfer was also evaluated.

MATERIALS AND METHODS

Cell lines

Five human colon cancer cell lines HT29, SW480, SW948, SW1116 and LS180, as well as the human embryonic kidney (HEK) cell line (CAR-positive) and Chinese hamster ovary (CHO) cells (CAR-negative) (ATCC numbers: HTB-38, CCL-228, CCL-237, CCL-233, CL-187, CRL-1573, CCL-61) were obtained from the National Cell Bank of Iran (NCBI). Cells were cultured in RPMI 1640, Dulbecco's Modified Eagle's Medium, Minimum Essential Medium, or L-15 medium. All cells were maintained with 100 U/mL penicillin, 100 μg/mL streptomycin and 100 mL/L fetal bovine serum (FBS). Cell lines were cultured either in 10-cm culture plates or in flasks at 37℃ and in 50 mL/L CO_2 in a humidified incubator.

RT-PCR

Total cellular RNA was extracted from each cell line by Biozol reagent (Bioflux, Japan). Two micrograms of total RNA were converted to cDNA by reverse transcription, using Moloney murine leukemia virus reverse transcriptase (Fermentas, Lithuania) and random primers (Roche, Germany) as described previously[23], with minor modifications. Since pseudogenes have been identified for the CAR gene, the CAR mRNA sequence was aligned with the pseudogenes (multiple alignment at http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.htm). CAR primers (CARf, 5'-CGTGCTCCTGTGCGGAGTAGT-3'; CARr, 5'-GACCCATCCTTGCTCTGTGCT-3') were designed in exons 1 and 7 with intervening introns of approximately 51 Kb, in a way that would align only with CAR mRNA and mismatched the pseudogenes in at least one nucleotide at the 3'-ends. Phosphoglucomutase-1 (PGM-1), human house keeping gene primers (PGMf, 5'-TCCGACTGAGCGGCACTGGGAGTGC-3'; PGMr, 5'-GCCCGCAGGTCCTCTTTCCCTCACA-3') or murine β-actin house keeping gene primers (actinf, 5'-GAACCCT AAGGCCAACCGTGA-3'; actinr, 5'-AGGAAGAGGAT GCGGCAGTGG-3') were used as internal controls.

The PCR protocol consisted of an initial denaturation for 5 min at 94℃; followed by 32 cycles of denaturation for 30 s at 94℃, annealing for 30 s at 62℃, and extension for 60 s at 72℃. The final cycle had a prolonged extension time of 7 min at 72℃. PCR products were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. The length of the expected product was 1068 (α-transcript) or 806 (β-transcript) bp for the CAR gene and 382 and 374 bp for the PGM1 and β-actin genes, respectively.

Cloning

Amplification products of the CAR β-transcript were cloned using InsTAclone PCR Cloning Kit (Fermentas) following the manufacturer's instructions. In brief, PCR products were ligated into a pTZ57R/T vector and transformed by a heat shock method into competent *Escherichia coli*. Transformants were selected in LB medium containing ampicillin, X-gal and IPTG. The presence of the expected insert was confirmed by PCR and sequencing.

Immunoblotting

For immunoblotting analysis, cells were lysed for 1 h at 4℃ in lysis buffer containing 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 10 mL/L Nonidet-P40 (NP40), 0.1 mL/L SDS and one tablet of complete, mini, EDTA-free protease inhibitor cocktail (Roche, Germany) per 10 mL lysis buffer. After the removal of cell nuclei by centrifugation, protein concentrations were measured by the Bradford protein assay. Equal amounts of protein (15 μg) from each cell lysate were diluted with SDS loading buffer, heated for 5 min at 95℃ and electrophoresed on a 10% SDS-polyacrylamide gel. The separated proteins were then electrotransferred to a nitrocellulose membrane, which was blocked with 50 g/L non-fat dried milk in Tris buffered saline-Tween (TBST) buffer to block non-specific binding sites. The blot was then incubated with polyclonal rabbit anti-CAR primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1/1000 dilution in TBST and 0.01 g/mL BSA) with gentle shaking for 1 h at room temperature. After extensive washing, polyclonal anti-rabbit horseradish peroxidase-conjugated secondary antibody (1/1000 dilution in TBST and 0.01 g/mL BSA) was applied for 1 h at room temperature with gentle shaking. Bands were visualized using an ECL kit (Amersham) according to the manufacturer's instructions.

Adenoviral vector

Adenoviral vector expressing the green fluorescent protein (GFP) gene driven by the cytomegalovirus promoter[24] was used to assess the colon cancer cell lines infected with adenoviral vector. Virus titer was determined by optical absorbance at A_{260} (1 A_{260} unit = 10^{12} particles/mL).

Adenovirus-mediated GFP gene transfer

To evaluate reporter gene transfer by the adenovirus vector, five colon cancer cell lines were plated in 24-well culture plates at a density of 5×10^4 cells/well and grown at 37℃ in a humidified incubator. After overnight incubation, cells were infected with Ad5-GFP at a multiplicity of infection (MOI) of 50 virus particles/cell. Two hours later, cells were washed twice with PBS and 600 μL fresh complete medium was added to every well. After 36 h, cells were evaluated for GFP expression by fluorescence microscopy.

RESULTS

CAR mRNA expression in colon cancer cell lines

We first examined the expression of CAR by RT-PCR. Integrity was evaluated by running RNA on 1% agarose gel and the quantity of RNA was measured spectrophotometrically. cDNA was then synthesized and CAR specific sequences amplified using CAR-specific primers (CARf, CARr). As shown in Figures 1A and 2, CARf and CARr primers generated a seven-exon-encompassing fragment with the expected length of 1068 bp in all the colon cancer cell lines. These primers could not amplify the pseudogenes (Figure 2, lane 1), and therefore the bands observed in lanes 2-6 were generated by just the amplification of cDNA. Any probable

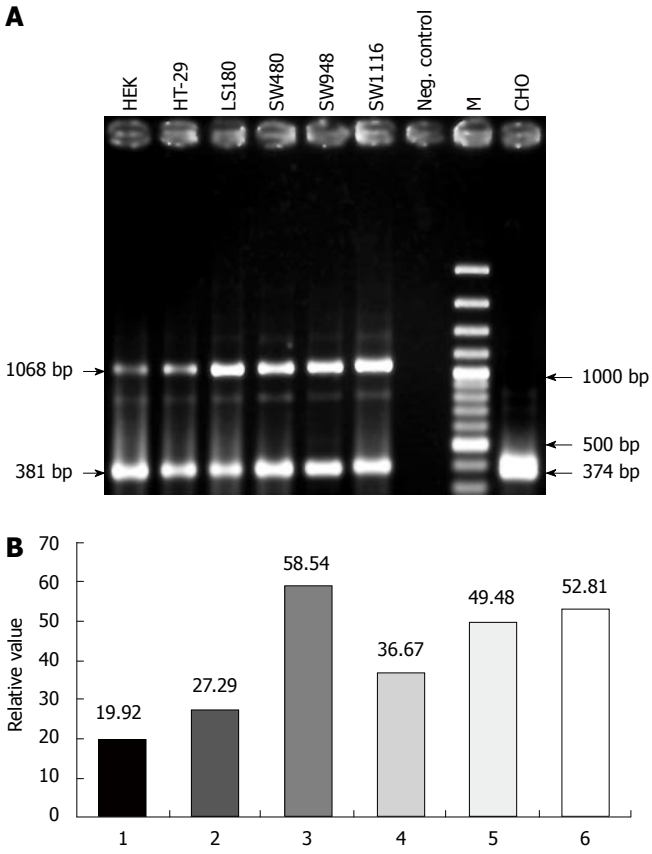

Figure 1 **A**: Multiplex RT-PCR of expression of coxsackievirus and adenovirus receptor, PGM and β-actin (internal controls); **B**: CAR mRNA level in different cell lines. 1-6 refer to HEK (positive control), HT-29 (colon), LS180 (colon), SW480 (colon), SW948 (colon) and SW1116 (colon) cells, respectively.

contamination of cDNA with genomic DNA could be disregarded.

CAR protein expression in colon cancer cell lines

We examined the expression of CAR by Western blotting. For immunoblotting, cell lines were cultured, harvested, lysed in appropriate lysis buffer and finally analyzed with anti-CAR antibodies. As shown in Figure 3, a band of about 46 ku was detected in HEK, HT-29, Ls-180, SW480, SW948 and SW1116 cell lines but was not observed in the negative control CHO cells.

Alternatively spliced variant of CAR

After electrophoresis and staining of RT-PCR products, an additional fragment of 806 bp was also observed (Figure 2). This shorter band produced by CAR-specific primers was cloned and sequenced, which revealed that this fragment was the result of an alternative splicing event between exons 4 and 7, lacking exons 5 and 6, of the CAR normal transcript, and was therefore the previously determined β-splice variant. All the colon cell lines studied expressed the β-splice variant although the band intensity of the splice variant was low when compared with the intensity of the normal transcript.

Detection of GFP gene expression in colon cancer cell lines

As shown in Figure 4, Ad5-GFP vector entered the cells and expression of the GFP gene indicated the efficient infection of these cells by the adenoviral vector.

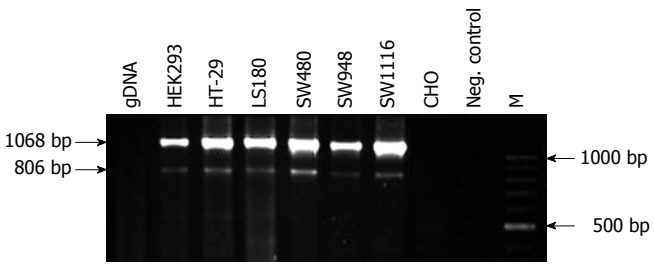

Figure 2 Expression of CAR in colon cancer cell lines. Two micrograms of total RNA prepared from several different human colon cancer cell lines were subjected to RT-PCR analysis. RT-PCR revealed β-transcript expression in addition to the normal CAR gene transcripts.

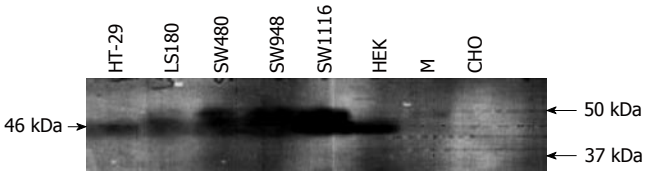

Figure 3 Western blot analysis of CAR protein in colon cancer cell lines. Total protein from five human colon cancer cell lines was separated by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with the correct anti-CAR antibodies.

DISCUSSION

Gene therapy is a therapeutic modality for malignant cancers. For this, the recombinant adenovectors Ad2 and Ad5 derived from the human adenovirus subgroup C are attractive gene transfer vehicles for cancer gene therapy[25,26]. As therapeutic efficacy has been demonstrated to correlate with the ability of adenovirus to enter target cells, expression of CAR has been studied extensively.

Several tumor cell lines including those for ovary cancer[17], head and neck carcinoma[14], renal cell carcinoma[15], prostate[21] and bladder[16] cancer have shown relatively low CAR expression; although high expression of CAR mRNA has been reported in osteosarcoma cell lines[27]. In the present study, CAR expression was investigated in five colon cancer cell lines, and it was shown that CAR was expressed in all of them, although some variations in band intensity of RT-PCR products were observed among the different cell lines (Figure 1B). The mismatch between specific primers and CAR pseudogenes made us certain that only cDNA could act as the source of amplification in RT-PCR. The existence of CAR pseudogenes has apparently not been taken into account in previous studies that have investigated CAR expression[18,19,29].

Alternatively spliced products of the CAR gene have been identified; β-variant mRNA has been detected in multiple human tissues such as heart, brain, lung, liver, kidney and pancreas[10], and has also shown to be expressed in osteosarcoma cell lines[27], HeLa cells[28] and musculoskeletal tumors[29]. The β-transcript is predicted to encode a 252-amino-acid protein that can be released

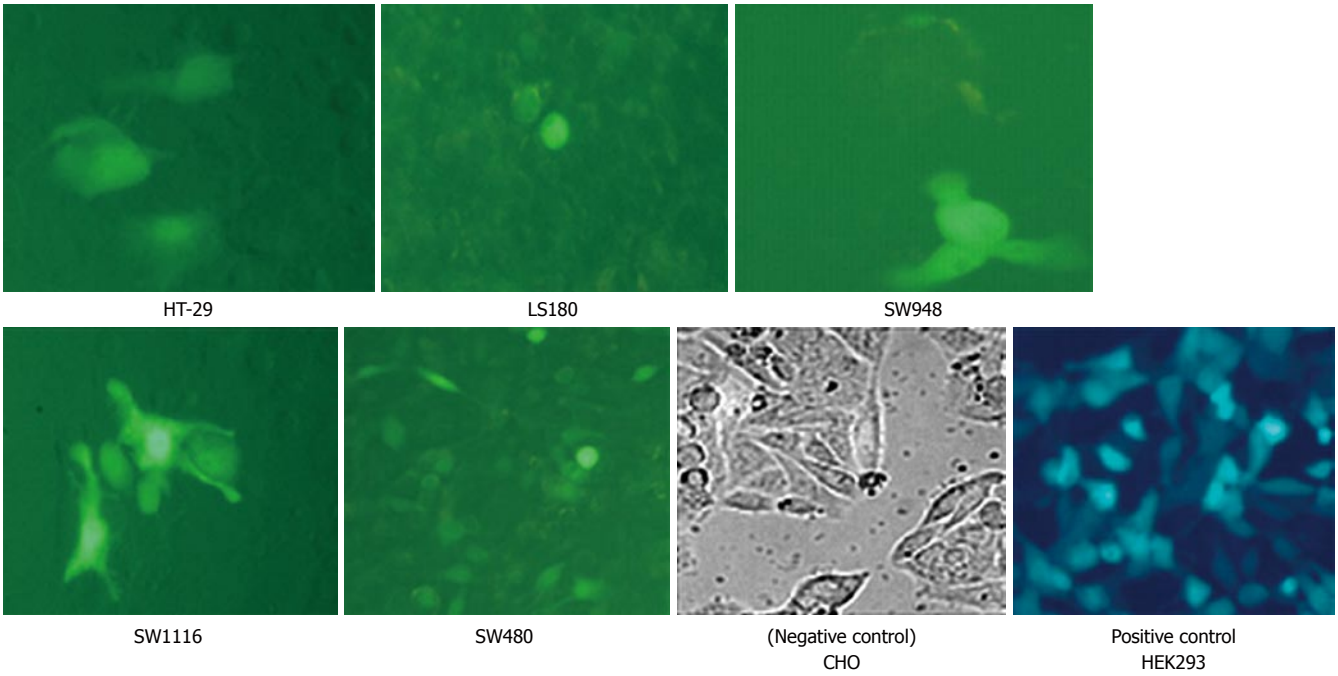

Figure 4 Sensitivity of colon cancer cells to adenovirus infection. Cells were plated in 24-well culture plates and infected with Ad5-GFP at an MOI of 50. After 36 h, cells were evaluated for GFP expression by fluorescence microscopy. GFP was expressed when recombinant adenoviruses infected the cells.

from cells to the culture medium because of the absence of a transmembrane domain[28]. CAR cytoplasmic and transmembrane domains are not necessary for virus attachment[30], so the anticipated β-variant protein might be as effective as the full-length variant in binding to adenovirus. In the present study, the β-transcript was demonstrated to be present in colon cell lines, in addition to the normal α-transcript.

To evaluate whether CAR mRNA expression correlated with CAR protein expression, Western blotting was carried out. CAR protein was shown to be present in the colon cell lines investigated; therefore, they could be considered as CAR-positive on the basis of CAR protein expression. In addition, no alternative protein band that could be attributed to the β-transcript-encoded protein was observed. Western blotting was carried out using a commercial polyclonal antibody against amino acids 1 to 300 of the original protein and cell lysates. Thus, the absence of any alternative signal (β-variant protein) could be explained as very low or undetectable intracellular level of this shorter variant and its secretion from the cell surface. However, β-variant protein is not detected in extracts of HeLa cells that express both β- and normal variants[28]. Therefore, the potential translation of CAR alternative transcript and its proposed regulatory role in antivirus defense mechanisms[28] remains to be confirmed.

Experiments with the Ad5 vector carrying the GFP reporter gene showed that the colon cancer cell lines studied were sensitive to adenovirus infection, and after 36 h GFP gene expression was easily detected by fluorescence microscopy.

In conclusion, the results of this study suggested that adenovirus-based gene delivery was efficiently applied to the five colon cancer cell lines. However, to clarify the efficiency of adenoviral-mediated gene transfer in colon tumors, further studies will be required to explore the expression of CAR.

COMMENTS

Background

Vectors based on human adenovirus serotypes 2 and 5 show increasing promise as gene delivery vehicles, and currently adenoviral vectors are the most commonly administered vectors for cancer gene therapy. Adenovirus initiates gene transfer by attachment to cell surface receptor, the coxsackievirus and adenovirus receptor (CAR).

Research frontiers

Reports show that the efficiency of adenoviral infection is dependent on the expression of CAR on target cells. However, adenovirus-mediated gene transfer is in practice hindered by the relatively low expression of CAR on tumor cells.

Innovations and breakthroughs

This is believed to be the first report to examine the expression of CAR in human colon cancer cell lines at the mRNA and protein level.

Applications

These results might be of potential value in adenovirus-based gene transfer and research in colon cancer cell lines.

Terminology

Gene therapy: to replace a malfunctioning mutated gene with a normal wild-type gene or to express a therapeutic gene in target cells. A prerequisite for efficient gene therapy is to determine a system for delivering the therapeutic gene to target cells. Adenovirus: non-enveloped, icosahedral, particles with double stranded DNA genome that in humans, is divided into six species (A-F) and 51 serotypes. CAR: primary cellular receptor for attachment of both coxsackie B virus and adenovirus subgroup C to target cells.

Peer review

This manuscript by Yassan Abdolazimi and co-authors is presented in a clear style.

Experimental details are provided accordingly. Relevant controls were included in the study. The authors' findings are discussed with the relevant literature.

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BASIC RESEARCH

Probiotic bacteria change *Escherichia coli*-induced gene expression in cultured colonocytes: Implications in intestinal pathophysiology

Pinaki Panigrahi, Gheorghe T Braileanu, Hegang Chen, O Colin Stine

Pinaki Panigrahi, Gheorghe T Braileanu, Departments of Pediatrics, University of Maryland School of Medicine, 22 South Greene Street, Baltimore, MD 21201, United States
Hegang Chen, Department of Epidemiology & Preventive Medicine, University of Maryland School of Medicine, 22 South Greene Street, Baltimore, MD 21201, United States
O Colin Stine, Genomics Core, University of Maryland School of Medicine, 22 South Greene Street, Baltimore, MD 21201, United States
Supported by the Department of Pediatrics and GCRC (M01-RR-16500), University of Maryland Baltimore, with partial funding from NIH grants UO1 HD 40574 and RO1 HD 053719
Correspondence to: Dr. Pinaki Panigrahi, University of Maryland School of Medicine, Department of Pediatrics, 22 South Greene Street, N5W68, Baltimore, MD 21201, United States. ppanigrahi@pol.net
Telephone: +1-410-7061803 Fax: +1-410-7060404
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Abstract

AIM: To investigate the change in eukaryotic gene expression profile in Caco-2 cells after infection with strains of *Escherichia coli* and commensal probiotic bacteria.

METHODS: A 19 200 gene/expressed sequence tag gene chip was used to examine expression of genes after infection of Caco-2 cells with strains of normal flora *E. coli*, *Lactobacillus plantarum*, and a combination of the two.

RESULTS: The cDNA microarray revealed up-regulation of 155 and down-regulation of 177 genes by *E. coli*. *L. plantarum* up-regulated 45 and down-regulated 36 genes. During mixed infection, 27 genes were up-regulated and 59 were down-regulated, with nullification of stimulatory/inhibitory effects on most of the genes. Expression of several new genes was noted in this group.

CONCLUSION: The commensal bacterial strains used in this study induced the expression of a large number of genes in colonocyte-like cultured cells and changed the expression of several genes involved in important cellular processes such as regulation of transcription, protein biosynthesis, metabolism, cell adhesion, ubiquitination, and apoptosis. Such changes induced by the presence of probiotic bacteria may shape the physiologic and pathologic responses they trigger in the host.

Key words: *Lactobacillus*; *Escherichia coli*; Gene expression; Probiotic; cDNA microarray

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INTRODUCTION

There has been an upsurge in clinical trials involving probiotics in gastrointestinal diseases. Although promising, these trials with specific probiotic bacteria have shown variable results, with limited elucidation of the underlying pathophysiology. In real life, these strains never act on the host cells in isolation and over 800 bacterial strains in the adult human colon are engaged in constant cross talk with intestinal epithelial cells. No detailed study so far has attempted to examine the effect of individual probiotic bacteria on host gastrointestinal cells, and the changes during co-infection with other enteric bacteria.

However, a lot of emphasis has recently been given to the normal bacterial flora in the intestine, including many *Lactobacillus* strains that are considered as probiotics with health-promoting effects on the host. A myriad of effects have been shown by these bacteria, spanning from bacterial killing *via* secretion of bacteriocins[1], to inhibition of attachment and invasion by pathogenic bacteria[2], and modulation of host inflammatory responses[3]. These commensal strains have been shown to modulate the expression of genes involved in important physiologic functions such as postnatal intestinal maturation, cell growth, proliferation, nutrient absorption, mucosal barrier function, and angiogenesis[4-6]. Multiple laboratory studies have shown beneficial effects of *Lactobacillus* strains against single pathogenic bacterial strains in *in vitro* and *in vivo* systems[7,8]. During the last few years, there has been an exponential increase of clinical trial reports and reviews in the literature pertaining to the utility of probiotics in gastrointestinal and allergic diseases[9-21]. Many small studies utilizing *Lactobacillus* and *Bifidobacteria* have shown beneficial effects such as better weight gain and improved

feeding tolerance[22] in neonates, and efficacy against neonatal necrotizing enterocolitis (NEC)[23-25] and sepsis[24]. Other reports have demonstrated no effect in NEC[26], and in some cases, deterioration of specific conditions with probiotic therapy[25]. Results of clinical trials done by our group have shown a wide range (0%-60%) of colonization rates in newborn infants when three different probiotic strains were used[27]. These mixed and non-reproducible results have raised more questions than providing answers, and have strongly suggested complex interactions among bacterial strains and epithelial cells in the human intestine[7,28-30].

At this time, our understanding of the response of eukaryotic cells (e.g., intestinal cells) is limited to nutrients and local factors[31], and virulence mechanisms involving individual microorganisms. Although contrasting signal transduction mechanisms in bacterial and eukaryotic gene transcription have been described[32], reports on cross talk between bacteria and epithelial cells have focused on single bacterial strains[33]. As a result, the physiologic and pathologic changes in the host cells as a response to multiple bacteria have not been addressed. Since the mammalian gut is colonized with multiple bacterial strains very quickly after birth, it is conceivable that the ultimate effect of probiotic treatment will depend greatly on the presence of other bacteria in the host intestine at that time.

In the current study, we examined the difference between gene expression in intestinal cells in response to infection with a single bacterial strain, compared to that during mixed infection. Caco-2 cells were utilized to discern the effect of *Lactobacillus* plantarum (the most common *Lactobacillus* species in humans)[34], *Escherichia coli* (a common Gram-negative enteric strain) and the combination of the two strains. A high-density cDNA glass microarray and standard techniques were employed to identify bacteria-induced gene expression in this eukaryotic system.

MATERIALS AND METHODS

Caco-2 cell culture model

Caco-2 cells, obtained from American Type Culture Collection (ATCC HTB-37), were used at passage 10-12. This human colon-adenocarcinoma-derived cell line has been used extensively for physiologic and enteric bacterial pathogenesis studies[35]. The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37℃ in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA with 10% fetal calf serum (Sigma, St. Louis, MO, USA), 2 mmol/L glutamine, 1.0 mmol/L sodium pyruvate, 0.1% non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. All experiments were performed without serum or antibiotics in 8-10-d-old cells after they reached confluence.

Bacterial strains

E. coli strain 6-1 was isolated from a healthy infant, and has been used previously in *in vitro* and *in vivo* studies in our laboratory[36]. This strain does not possess any known virulence genes[37]. We used a human strain of *L. plantarum* (ATCC 202195), the species most commonly isolated from humans[34].

Defined bacterial treatment of epithelial cells

Cells were washed in PBS and re-fed with experimental DMEM without serum or antibiotics before the experiments. Following previously described methods in which a maximal effect of *Lactobacillus* was seen, Caco-2 cells were infected with *E. coli* and/or *L. plantarum* at 1:10 multiplicity of infection, and incubated for 2 h[38].

cDNA microarray

For examination of Caco-2 cell gene expression under our experimental conditions, we used a high-density glass microarray H19K (University Health Network Microarray Centre, Toronto, www.microarrays.ca/home.html) that had 19 200 genes/expressed sequence tags (ESTs). These included fully characterized, partially characterized and some uncharacterized human gene elements. Each gene/EST was printed in duplicate in this array. The genes in the array represented constitutively expressed genes/ESTs and the manufacturer did not include genes that are transiently expressed, such as cytokines and chemokines. In our experiments, we used dye swapping procedures and bioinformatics tools considered as standard techniques that have been reported in similar studies in the past[39].

Sample preparation

Total RNA was extracted from Caco-2 cells grown in 75-cm^2 tissue culture flasks using the TRIZOL method (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. RNA samples were treated with RNAse-free DNAse to remove contaminating genomic DNA, examined by 260/280 nm UV absorption ratio (> 1.8) followed by assessment of integrity by running in a 1.2% agarose gel and ethidium bromide staining.

Preparation of fluorescent-labeled cDNA[40], hybridization[41] and signal detection

Total mRNA (10 μg) was reversely transcribed using 20 mmol/L dNTP mix including amino-allyl dUTP (AA-dUTP; Sigma) and 400 U SuperScript Ⅱ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The resulting aa-cDNA, cleaned with a QIAquick column (Qiagen, Valencia, CA, USA), was coupled with Cy3 or Cy5 dye (Amersham Biosciences, Piscataway, NJ, USA) in the presence of sodium bicarbonate for 1 h in the dark. After adding 10 μL 4 mol/L hydroxylamine and 125 μL buffer PB (Qiagen supplied) to each, the control and treatment samples were combined and cleaned using another QIAquick column. The elute was transferred to a Microcon YM 30 centrifugal filter device (Amicon Millipore, Bedford, MA, USA), and after adding 20 μL cot-1 human DNA (Gibco-BRL), the whole volume was concentrated to 5 μL. Ten microliters of 1 μg/μL poly (A) RNA (Sigma), 1 μL 10 μg/μL tRNA (Gibco-BRL), 4 μL water and 5 μL hybridization buffer (50% formamide, 5 × SSC (3 mol/L sodium chloride, 0.3 mol/L sodium citrate and 0.1% SDS) were added. The array was pretreated at 42℃ for 1 h with hybridization buffer. After overnight

Table 1 Common genes induced by bacterial treatment (Seventeen genes were influenced by both *E. coli* and *L. plantarum* and four genes by both *E. coli* and combination treatment)

Nr.	Gene symbol	Gene ID NCBI	Gene name	Location	Function	Relative fold modification		
						L.p.	E.c.	Mix
1	GPR34	2857	G protein-coupled receptor 34	Integral to plasma membrane	G-protein coupled receptor activity	2.43	3.03	-0.53
2	GTPBP4	23560	GTP binding protein 4	Nucleus	Ribosome biogenesis - small GTPase mediated signal transduction	2.00	2.91	-0.30
3	TFPI2	7980	Tissue factor pathway inhibitor 2	Extracellular matrix	Serine-type endopeptidase inhibitor activity	2.10	2.93	-0.27
4	CYP26A1	1592	Cytochrome P450, family 26, subfamily A, polypeptide 1	Membrane	Metal ion binding	2.31	2.39	-0.88
5	ZNF35	7584	Zinc finger protein 35 (clone HF.10)	Nucleus	Transcription factor activity	2.18	2.19	-0.66
6	RTTN	25914	Rotatin		required for left-right specification in mouse embryos	2.21	2.13	-0.58
7	FXYD3	5349	FXYD domain containing ion transport regulator 3	Membrane	Chloride channel activity	2.44	2.03	-0.15
8	CYYR1	116159	Cysteine/tyrosine-rich 1	Integral to membrane	Molecular function unknown	-2.30	-2.20	1.05
9	BFAR	512836	Bifunctional apoptosis regulator		Apoptosis regulator	-2.40	-2.17	1.06
10	C19orf4	25789	Chromosome 19 open reading frame 4	Integral to membrane	Molecular function unknown	-2.51	-2.28	0.19
11	KIAA1305	57523	KIAA1305 protein		Hypothetical protein	-2.19	-2.29	0.55
12	PCDH9	5101	Protocadherin 9	Integral to membrane	Cell adhesion	-2.18	-2.30	0.50
13	IKIP	121457	IKK interacting protein			-2.23	-2.33	0.83
14	FLJ21963	79611	FLJ21963 protein		Hypothetical protein	-2.12	-2.37	1.88
15	SCRG1	11341	Scrapie responsive protein 1	Extracellular space	Nervous system development	-2.14	-2.54	0.74
16	ULK2	9706	Unc-51-like kinase 2 (C. elegans)		Similar to a serine/threonine kinase in C. elegans	-2.46	-2.72	0.26
17	LIFR	3977	Leukemia inhibitory factor receptor	Integral to plasma membrane	Receptor activity	-2.26	-2.96	0.29
18	BMF	90427	Bcl-2 modifying factor	Sequestrated by myosin	Apoptotic activator - protein binding	1.00	2.95	2.40
19	CD248	57124	CD248 antigen, endosialin		Marker of stromal fibroblasts	0.74	2.31	2.10
20	PPM1E	22843	Protein phosphatase 1E (PP2C domain containing)		Phosphatase	0.76	2.33	2.06
21	CARD8	22900	Caspase recruitment domain family, member 8		Involved in NFκB pathway	-0.59	-3.26	4.07

E.c., *E. coli*; L.p., *L. plantarum*.

hybridization at 42℃, the slides were washed in 50 mL 2 × SSC and 0.1% SDS at 55℃ for 5 min, once in 0.1 × SSC and 0.1% SDS for 5 min at room temperature (RT), and for 5 min with 0.1 × SSC at RT, air-dried and scanned with 555 nm and 647 nm lasers in a Scan Array 5000 (GSI Lumonics, Novi, MI, USA). Images of the fluorescence intensity for each dye were analyzed using Imagene 4.2 software (Biodiscovery, CA, USA).

RNA from each experimental condition and control Caco-2 cells were hybridized on the same microarray. To eliminate the color bias, duplicate reactions were carried out in which the dyes (Cy3, Cy5) for the control and experimental samples were swapped.

Data interpretation

Individual gene intensity data files for each experimental condition were compared with the control values using the GeneSight 2.1 program (Biodiscovery). After correction for the local background, normalization using all the spots, removal of the outliers, averaging of the replicates and transforming to base 2, each gene was assigned a relative expression value when compared with the control. A twofold or larger difference in the relative gene expression was considered significant.

The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE5874.

Real-time quantitative PCR

We randomly selected eight genes (BMF, CD248, PPM1E, FXYD3, OAS2, FY, CERK and HPSE) from our pool of expressed genes/ESTs that are well characterized in the literature and appear to have some biologic significance. ESTs were not included. Real-time quantitative PCR (Bio-Rad iQ SYBR Green Supermix and iCicler) was done using GAPDH for normalization. The levels of expression detected by microarray were compared with PCR results. The primers used to amplify specific gene segments are presented in Table 1. The relative gene expression was calculated using the comparative $\Delta\Delta C_T$ method. Each sample was tested twice in triplicate.

RESULTS

Gene expression after bacterial infection

After 2 h treatment, *E. coli*, *L. plantarum* and their combination changed the expression (by twofold) of 332,

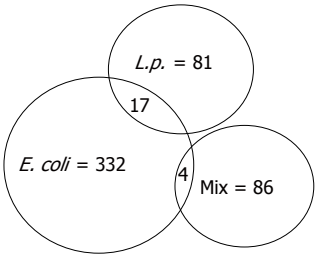

Figure 1 Schematic representation of the genes influenced by each treatment, and the overlapping (common) genes among treatments. The relative gene expression after treatment was > 2-fold compared with the control.

81 and 86 genes, respectively, compared to uninfected control Caco-2 cells (Figure 1). After infection with *E. coli*, 155 genes were up-regulated and 177 were down-regulated (Table 1 and Supplementary Table 1). *L. plantarum* induced up-regulation of 45 genes and 36 genes were down-regulated (Table 1 and Supplementary Table 2). The combination treatment up-regulated 27 genes and down-regulated 59 (Table 1 and Supplementary Table 3) [Note: The supplementary tables above can be accessed at: http://panigrahipeds.googlepages.com/suppl-tables.pdf; Raw data of all 19 200 genes during each treatment can be accessed from the NCBI/GEO data base (GSE5874) at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nzyxdkkuwukuytk&acc=GSE5874]. Mixed infection nullified the previously demonstrated stimulatory and inhibitory effects of *E. coli* on 152 and 177 genes and of *L. plantarum* on 38 and 26 genes, respectively. Stimulation of 23 and inhibition of 59 genes were noted after mixed infection that was not influenced by either bacterium alone.

There were 21 genes influenced by two different treatment conditions (Table 1). Seventeen genes were affected by *E. coli* and *L. plantarum*, and four by *E. coli* and the combination of bacteria. Genes nos. 1-7 were up-regulated by both *E. coli* and *L. plantarum*; and genes nos. 8-17 were down-regulated by both bacteria. For each of the 17 genes in this group, the effects of the individual bacteria were brought to baseline by the combination treatment. In contrast, for three genes BMF, CD248 and PPM1E (nos. 18, 19 and 20 in Table 1), the stimulatory effect of *E. coli* was maintained after mixed infection with *L. plantarum*. For one gene (no. 21, CARD8), the 3.26-fold down-regulation by *E. coli* was reversed in the mixed infection, with demonstration of a four fold increase.

Apart from the specific up- and down-regulation of genes by either *E. coli* or *L. plantarum*, and reversal of *E. coli*-induced effects when *L. plantarum* was used as a co-infectant, several genes of physiologic importance were noted in our system. Table 2 describes 58 genes under 10 specific categories that were expressed during mixed infection. While the function of a small number of genes was not very well defined, most of the genes could be grouped into important cellular functions. These include genes involved in transcription regulation, RNA processing, protein biosynthesis, and other important processes such as ubiquitination, cell adhesion, proliferation and apoptosis.

Confirmation of selected gene expression by real-time quantitative PCR

Eight genes were randomly tested by quantitative real-time PCR to verify the expression detected by microarray (Table 3). For each of these genes, RT-PCR confirmed their expression after the three bacterial treatments in the same direction (stimulation or inhibition) as in the microarray experiments (Figure 2).

DISCUSSION

The infant gut is essentially sterile at birth and is first colonized with Enterobacteriaceae, which change the redox potential in the intestine and allow more microaerophilic and anaerobic species to colonize[42,43]. The latter group, which is comprised primarily of *Bifidobacteria* and *Lactobacillus* organisms[44], are considered as normal flora that coexist in the human colon, as new species are introduced to ultimately provide a stable flora in the human gut[45], in which over 800 bacterial species coexist in harmony[46]. In such a healthy state, the intestinal mucosa serves as the first line of defense against infections by providing an important mechanical and immunologic barrier between the host's internal milieu and the gut environment. These intestinal epithelial cells generate and transmit signals between bacteria and deeper layers in the intestine[47]. In the event of specific infections, epithelial cells express and secrete proinflammatory and chemoattractant cytokines[48] that further transmit signals to the underlying cells in the reticuloendothelial system[47]. The virulence factors and the host responses to these factors in various diseases have been studied in a fair amount of detail (*E. coli*, *Vibrio cholerae*, *Salmonella* and *Pseudomonas*) using tissue culture and *in vivo* models, and specific genes and gene functions have been described[49-52]. These experiments have utilized single bacterial strains.

In an attempt to mimic the natural gut environment, communication systems among bacteria have also been studied relatively well. Chemical signals produced and detected by bacteria can be directed at other bacteria and self. This phenomenon, called as quorum sensing, is important for the microorganism's adaptation to the local environment[53]. This fundamental prokaryotic behavior (among bacteria) is known to affect the symbiotic or antagonistic environment created within the gut milieu. However, the effect of single versus multiple bacterial species on eukaryotic cells has not been addressed in the literature.

The stimulus for us to conduct the current study came from our observation that a large number of probiotic trials have been conducted and reported in the recent past, with almost no basis for selection of the strain, and more importantly, with no data on changes in physiologic or pathologic parameters in the host, other than analysis of the primary and secondary clinical endpoints. Although a live bacterial supplement was used in all of these reported studies, there was also a serious lack of data on the colonizing ability of the probiotic strain and changes in the colonization by other bacteria in the host gut. Since the newborn gut is colonized with a paucity of bacteria (an average 2.5 species in preterm infants)[37,54] that expands to a limited but heterogenous flora by 10 d of age[55], we designed the current simple system to examine the effects of *L. plantarum*, a common human probiotic strain, and *E. coli*, the most common colonizing strain in the neonatal

Table 2 Modulation of gene expression during mixed (*E. coli* and *L. plantarum*) infection

Biological process	Gene symbol	Gene ID NCBI	Gene name	Fold change
Category 1:	HOXD10	3236	Homeobox D10	2.50
Regulation of transcription	PHF7	51533	PHD finger protein 7 (Zinc ion binding)	2.44
	EGR1	1958	Early growth response 1	-2.08
	TRIM24	8805	Tripartite motif-containing 24 (Zinc ion and DNA binding)	-2.15
	ENO1	2023	Enolase 1, (alpha) (DNA binding)	-2.34
Category 2: RNA processing	SSB	6741	Sjogren syndrome antigen B (autoantigen La)	-2.08
	FUSIP1	10772	FUS interacting protein (serine/arginine-rich) 1	-2.21
	NOLA5	10528	Nucleolar protein 5A (56 kDa with KKE/D repeat)	-2.27
	DDX5	1655	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	-2.71
Category 3:	NEURL	9148	Neuralized homolog (Intracellular protein transport)	2.91
Protein biosynthesis, folding,	WDR36	134430	WD repeat domain 36	2.37
binding and transport	MTFMT	123263	Mitochondrial methionyl-tRNA formyltransferase	2.04
	ARF4	378	ADP-ribosylation factor 4	-2.03
	CEP57	9702	Centrosomal protein 57 kDa	-2.11
	ETF1	2107	Eukaryotic translation termination factor 1	-2.27
	HSPA1A	3303	Heat shock 70 kDa protein 1A	-2.28
	LGALS3	3958	Lectin, galactoside-binding, soluble, 3 (galectin 3)	-2.75
	HSPH1	10808	Heat shock 105 kDa/110 kDa protein 1	-2.93
	HSPA8	3312	Heat shock 70 kDa protein 8	-2.97
Category 4: Structural protein	AMPH	273	Amphiphysin (Actin cytoskeleton)	3.04
	MAP1B	4131	Microtubule-associated protein 1B	2.13
	ABCC10	89845	ATP-binding cassette, sub-family C (CFTR/MRP), member 10	-2.05
	SLC26A2	1836	Solute carrier family 26 (sulfate transporter), member 2	-2.06
	TUBB2A	7280	Tubulin, beta 2A	-2.15
Category 5: Metabolism	C5orf14	79770	Chromosome 5 open reading frame 14	2.91
	NAV2	89797	Neuron navigator 2	2.83
	SLC24A4	123041	Solute carrier family 24 (sodium/potassium/calcium), member 4	2.35
	PLEKHM2	23207	Pleckstrin homology domain containing, family M, member 2	2.10
	TWF1	5756	Twinfilin, actin-binding protein, homolog 1 (Tyrosin kinase)	-2.01
	AKR1C1	1645	Aldo-keto reductase 1, member C1 (Bile acid binding)	-2.09
	HMGCR	3156	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-2.10
	DC2	58505	DC2 protein (Glycotransferase activity)	-2.12
	GSTA1	2938	Glutathione S-transferase A1	-2.15
	GAPD	2597	Glyceraldehyde-3-phosphate dehydrogenase	-2.16
	GCLC	2729	Glutamate-cysteine ligase, catalytic subunit	-2.21
	SRM	6723	Spermidine synthase	-2.39
	HSP90AA1	3320	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	-2.46
	AHCY	191	S-adenosylhomocysteine hydrolase	-2.48
	MAT2A	4144	Methionine adenosyltransferase Ⅱ, alpha	-2.74
Category 6: Cell physiology	NCF4	4689	Neutrophil cytosolic factor 4, 40 kDa	2.39
	CYCS	54205	Cytochrome *c*, somatic	-2.02
	DBI	1622	GABA receptor modulator, acyl-Coenzyme A binding protein	-2.25
	ATP5G3	518	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3	-2.26
	HSPA1L	3305	Heat shock 70 kDa protein 1-like	-2.26
Category 7: Cell proliferation	FOSL1	8061	FOS-like antigen 1 (transcription factor activity)	-2.09
	FGG	2266	Fibrinogen gamma chain	-2.36
	FGG	2244	Fibrinogen beta chain	-2.45
Category 8: Cell adhesion	NELL2	4753	NEL-like 2 (Calcium ion binding)	2.25
	ITGB3	3690	Integrin, beta 3 (platelet glycoprotein Ⅲa, antigen CD61)	2.11
	RHOB	388	Ras homolog gene family, member B	-2.19
	ADRM1	11047	Adhesion regulating molecule 1	-2.25
Category 9: Ubiquitination	UBE2N	7334	Ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	-2.02
	UBE2S	27338	Ubiquitin-conjugating enzyme E2S	-2.05
	ANAPC7	51434	Anaphase promoting complex subunit 7	-2.15
	CACYBP	27101	Calcyclin binding protein	-2.18
	UBA52	7311	Ubiquitin A-52 residue ribosomal protein fusion product 1	-2.29
	COL6A3	1293	Collagen, type Ⅵ, alpha 3	2.33
	RPS3A	6189	Ribosomal protein S3A	-2.08
	TWF1	5756	Twinfilin, actin-binding protein, homolog 1 (Tyrosin kinase)	-2.01
	AKR1C1	1645	Aldo-keto reductase 1, member C1 (Bile acid binding)	-2.09

Negative value indicates reduction in gene expression.

period, on gut cells. We took advantage of a microarray chip that allowed us to examine 19 200 human genes in this simulated microbial gut environment. In this *in vitro* model, single and combined bacteria were allowed to interact with cultured cells, and our results were analyzed under high-stringency conditions to identify specific genes expressed during defined bacteria-gut cell interactions.

In our system, we observed a change (up- or down-regulation) in the expression of 333, 81 and 86 genes upon infection with *E. coli*, *L. plantarum* and the combined

Table 3 Primers used for RT-PCR

Gene symbol	Gene ID (NCBI)	Gene name	Gene role	Primer	Primer sequence 5′-3′
BMF	90427	Bcl2 modifying factor, transcript variant 1	Has a single Bcl2 homology domain 3 (BH3), binds Bclk2 proteins and functions as an apoptotic activator	F	GCTTCAGTTGCATTGCAGACCAGTT
				R	AGAGCCCTTGGGAATTCTCACCAT
CD248	57124	CD248 antigen = endosialin	A gene regulated by the cell density *in vitro*. Has a calcium binding domain	F	TCAACTACGTTGGTGGCTTCGAGT
				R	AGTTGGGATAATGGGAAGCTGGGT
PPM1E	22843	Protein phosphatase 1E	Member of the PP2C family of Ser/Thr phosphatases known to be negative regulators of stress response pathways	F	ATGCCTCCATTCACCTCCACGTTA
				R	TGTCATAGAAGCCATCACAGGCCA
FXYD3	5349	FXY domain containing ion transport reg. 3	The protein encoded by this gene may function as a chloride channel or as a chloride channel regulator	F	AATGCAAGTTTGGCCAGAAGTCCG
				R	TTGCATATGAGGTCCCATGGCTGA
OAS2	4939	2′-5′-oligoadenylate synthetase 2	This enzyme family plays a significant role in the inhibition of cellular protein synthesis	F	AGAAGCCAACGTGACATCCTCGAT
				R	TGCTGGAGTTCAGTGAAGCAGACT
FY	2532	Duffy blood group antigen	Helps in leukocyte recruitment to sites of inflammation by facilitating movement of chemokines across the endothelium	F	TGACTCTGCACTGCCCTTCTTCAT
				R	TTGACAACAGCAACAGCTTGGACC
CERK	64781	Ceramide kinase	Integral to membranes, has roles in arachidonic acid release and production of eicosanoids	F	TGAGAAGAAACGGTGGTTGGGTCT
				R	AGCATTTCCGGATGAGGATGAGGT
HPSE	10855	Heparanase	Cell surface expression and secretion markedly promote tumor angiogenesis and metastasis	F	ACCTTTGCAGCTGGCTTTATGTGG
				R	CTTGCACGCTTGCCATTAACACCT
GAPDH	2597	Glyceraldehyde-3-phosphate dehydrogenase	Used as reference	F	GACCACAGTCCATGCCATCAC
				R	GAGCTTCAGAAAGTGGTCGTTGA

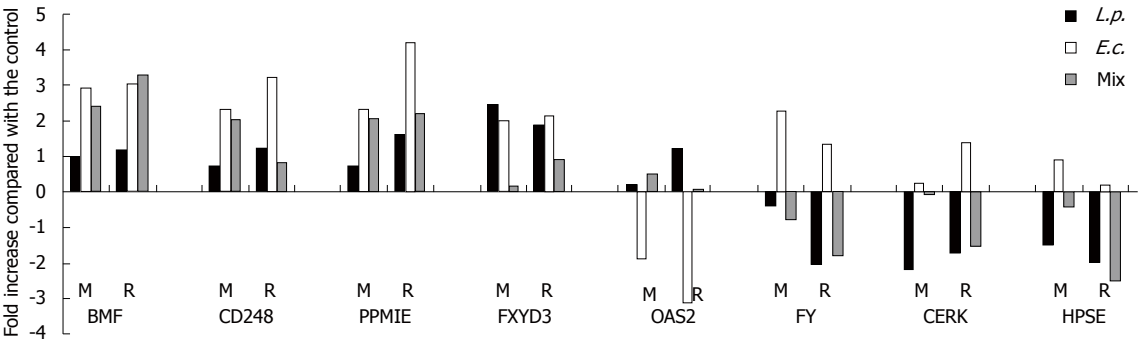

Figure 2 Effect of different bacterial treatments on expression of eight genes assessed by microarray (M) and RT-PCR (R).

treatment, respectively. Our real-time PCR experiments confirmed the modifications demonstrated in the microarray experiments, albeit at a lower level, a phenomenon also reported in other studies[50]. The numbers of unique genes presented in this study are in the range reported in previous studies in which Gram-negative enteric pathogens modified the expression of 0.5%-13% of the genes in epithelial cells[50,56-58], and commensal bacteria induced differential expression of 0.35%-6.2% of examined genes in mouse colonocytes[59]. Our strain of *E. coli* modified 1.73%, and *L. plantarum* modified 0.43% of genes. The slightly lower number of genes identified in our 19200 array may have been due to the use of a non-pathogenic strain of *E. coli*, a commensal *Lactobacillus*, and an array that included only constitutively expressed genes. Genes expected to be expressed after a bacterial insult such as pro-inflammatory cytokines were not spotted on this array. Additionally, a slightly low number might have resulted from our conservative choice of a twofold increase in expression as being significant in our analysis.

There are several comparisons that can be made between our results and those of others using a similar approach but with single bacterial infection. For example, from the six genes up-regulated by enteropathogenic *E. coli* in HeLa cells[49], we found only one (zyxin, a cytoskeletal protein) to be in common with our microarray results. There was a similar increase (1.72-fold) in expression of this gene when our *E. coli* strain 6-1 was used to infect Caco-2 cells. Two previous studies with commensal flora have reported that bacterial reconstitution of germ-free mice increased the expression of the colon-specific serum amyloid A1 gene[60,61]. In our model, serum amyloid A2 gene expression was increased by 2.22-fold. From the 12 genes down-regulated by non-pathogenic bacterial reconstitution of germ-free mice, reported by Fukushima *et al*[59] in colonic epithelial cells, three were in common with our microarray; selenoprotein P, 3-hydroxy-3 methylglutaryl-coenzyme A synthase and metallothionein. All three were also down-regulated in our combination treatment model. The authors also showed a down-regulation of solute carrier family 20 - member 1. Our results were very similar to this observation in that we also noted a decrease in the expression of other members of the solute carrier families, i.e., family 2, 9, 12, 20, 24, 25 and 35. Fukushima *et al*[59] have shown overexpression of heat shock protein (60 kDa) in germ-free mice compared to specific pathogen-free rodents that had received treatment with normal mouse flora. We observed a similar phenomenon in our system in which down-regulation of heat shock proteins 75, 105 and

an ortholog of mouse heat shock protein 70 kDa were noted after combined bacterial treatment. We observed cytochrome c oxidase subunits Ⅳ isoform 1, Va, Ⅵb, Ⅵc, Ⅶa, Ⅶb, Ⅶc and Ⅷ to be up-regulated after *L. plantarum* treatment, similar to that described by Hooper *et al*, who demonstrated up-regulation of cytochrome *c* oxidase subunit 1 by *Bacteroides*, another species also considered as commensal flora. Hooper and colleagues have also shown up-regulation of calmodulin after treatment with *Bacteroides*[61]. Similar increases in expression were noted for calmodulin 1, 2 and 3, calmodulin-dependent protein kinase and phosphodiesterase in our system.

We observed modulation of multiple genes known to have an impact on cellular and physiologic processes in the eukaryotic system (Table 2). These genes ranged from basic transcriptional regulators to those involved in protein synthesis, cellular metabolism, cell proliferation and apoptosis. During mixed infection, we observed down-regulation of three genes involved in ubiquitination. Ubiquitin-conjugating enzyme E2N, ubiquitin-carrier protein E2-EPF and ubiquitin A-52 residue ribosomal protein fusion product 1 were reduced 2.02, 2.05 and 2.29-fold, respectively. In a recent study that investigated anti-inflammatory properties of *Lactobacillus casei*, expression of several genes involved in ubiquitination was reduced, including E2N, a gene (common to our system) that was reported to be decreased 2.88-fold[62]. The authors concluded I-κB stabilization *via* reduced ubiquitination and downstream modulation of inflammatory response driven by NF-κB in *Shigella*-infected Caco-2 cells. We used a non-pathogenic commensal strain of *E. coli* in our experiments, and while the aim of the current study was not to assess or examine the effects of *L. plantarum* during bacterial infection or inflammation, our results strongly suggest that *Lactobacillus* strains do indeed affect common physiologic pathways in gut cells, which may ultimately shape the host response in health and disease.

In our study, it was important and intriguing to note that the three experimental infections induced quite unique gene-expression profiles. Even the mixed infection with *E. coli* and *L. plantarum* had a very small overlap with the expression profiles of the strains when they were used alone. This illustrates how colonization can change the gene expression of host cells as they are exposed to more than one species of bacteria. In real life, the gut cells are exposed to a multitude of bacterial strains, and hence, it may be of limited value to study the effect of infection or colonization by single bacterial species in a clean tissue culture environment, and use the results as the basis for designing treatment or preventive strategies. Using neonatal models of gut colonization, we have previously shown that bacterial ecology (combination of Gram-negative and Gram-positive organisms), rather than individual virulent bacterial strains, plays a more important role in diseases such as NEC[36]. The results of our current study are in line with previous observations, and now provide an additional line of support and offer a possible explanation for the varied results of recent probiotic trials. On a broader scale, this report provides an insight into the complex host response that can be expected at mucosal sites such as the gastrointestinal tract. Based on the results obtained from tissue culture with only two bacteria in the system, it can be speculated that our findings are only the tip of the iceberg, and the real *in vivo* picture in mammals will be even more complex. While it is becoming increasing clear that specific *Lactobacillus* species posses unique health-promoting characteristics[29], knowledge gained from the current study further indicates that a "one strain fits all" approach may not always succeed in the treatment or prevention of specific diseases. A more global approach needs to be taken with proper emphasis on the microbial ecology, while addressing the pathogenesis of unique bacterial diseases in the mammalian intestine at different ages and stages of development.

In the context of *in vivo* or clinical trial environment, it should be noted that our current model and results do not represent a universal phenomenon, nor provide a comprehensive picture of the human intestine. For example, genes expressed will probably be different if other probiotic strains such as *Bifidobacteria* and *L. casei* were used in our system. Similarly, combinations of other aerobic and anaerobic Gram-negative and Gram-positive strains may induce different sets of genes. We can utilize other microarray systems with cytokine and signaling-molecule genes (not spotted in the current 19 200 gene array), when our aim would be to identify modifications in inflammatory mediators. The relative concentrations of each bacterium in the system may also change the gene-expression profile. In the current study, we selected a 1:10 ratio of *E. coli* to *Lactobacillus* infecting dose to simulate the human intestinal microflora, in which anaerobic and microaerophilic organisms form the dominant flora[63]. Since enteric bacteria such as *E. coli* are sometimes present at < 0.1% of the total bacterial population, with a predominance by obligate anaerobes[64], it is not unexpected to observe a different gene-expression profile when a 10-100-fold higher proportion of *Lactobacilli* are used in the system. Nevertheless, such manipulations and experiments can be done, and despite some limitations, assessment of mRNA-expression profiles by cDNA array analysis can be utilized as a useful technique for expanding our understanding of the colonocyte-bacteria interaction[50].

While it may appear difficult to analyze complex microflora (400-800 species) and their interactions with gut cells in the mature intestine, this is now made feasible with the availability of new techniques. Fluorescent *in situ* hybridization utilizing bacterial rRNA can identify and quantify major genera of bacteria, even if they are non-culturable in stools[65,66]. Bacterial microarray chips developed during the last year can identify thousands of bacterial species in stools in one experiment[67,68]. Denaturing gradient gel electrophoresis can be utilized to monitor changes in microflora pattern[69,70] over time and after administration of probiotic supplements. Live colonocytes can be isolated from stool samples and used to examine the expression of genes and proteins during different experimental and/or disease states[71,72]. At this juncture, there is a need for the scientific community to engage in careful evaluation of probiotic strains in *in vitro* and *in vivo* systems prior to initiation of clinical trials. With the new non-invasive tools at hand, such preclinical

endeavors, coupled with concurrent examination of changes in the gut flora and host responses during clinical trials, hold great promise in discerning the difference between "snake oil" and "magic bullets" when it comes to the role of probiotic therapy in human medicine.

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BASIC RESEARCH

Inflammatory cytokines promote inducible nitric oxide synthase-mediated DNA damage in hamster gallbladder epithelial cells

Amane Kitasato, Yoshitsugu Tajima, Tamotsu Kuroki, Ryuji Tsutsumi, Tomohiko Adachi, Takehiro Mishima, Takashi Kanematsu

Amane Kitasato, Yoshitsugu Tajima, Tamotsu Kuroki, Ryuji Tsutsumi, Tomohiko Adachi, Takehiro Mishima, Takashi Kanematsu, Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
Correspondence to: Amane Kitasato, MD, Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. a-kit@bg8.so-net.ne.jp
Telephone: + 81-958-497316 Fax: + 81-958-497319
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Abstract

AIM: To investigate the link between chronic biliary inflammation and carcinogenesis using hamster gallbladder epithelial cells.

METHODS: Gallbladder epithelial cells were isolated from hamsters and cultured with a mixture of inflammatory cytokines including interleukin-1β, interferon-γ, and tumor necrosis factor-α. Inducible nitric oxide synthase (iNOS) expression, nitric oxide (NO) generation, and DNA damage were evaluated.

RESULTS: NO generation was increased significantly following cytokine stimulation, and suppressed by an iNOS inhibitor. iNOS mRNA expression was demonstrated in the gallbladder epithelial cells during exposure to inflammatory cytokines. Furthermore, NO-dependent DNA damage, estimated by the comet assay, was significantly increased by cytokines, and decreased to control levels by an iNOS inhibitor.

CONCLUSION: Cytokine stimulation induced iNOS expression and NO generation in normal hamster gallbladder epithelial cells, which was sufficient to cause DNA damage. These results indicate that NO-mediated genotoxicity induced by inflammatory cytokines through activation of iNOS may be involved in the process of biliary carcinogenesis in response to chronic inflammation of the biliary tree.

Key words: Biliary carcinoma; Inflammation; Inflammatory cytokine; Nitric oxide; Inducible nitric oxide synthase; DNA damage; Gallbladder epithelial cell; Hamster

INTRODUCTION

It is well known that chronic local inflammation increases the risk for cancer development in several organs, such as colon, lung, pancreas, and esophagus[1,2]. Similarly, biliary carcinoma develops under chronic inflammatory conditions involving the biliary epithelium in the setting of gallstone disease, congenital choledochal cyst, pancreaticobiliary maljunction, or primary sclerosing cholangitis[3,4]. Moreover, recent reports have described the occurrence of secondary biliary carcinomas in patients with persistent reflux cholangitis after bilioenterostomy, transduodenal sphincteroplasty, or endoscopic sphincterotomy for both benign and malignant diseases of the liver, bile duct, and pancreas[5-7]. However, the molecular mechanisms of biliary carcinogenesis as a consequence of chronic biliary inflammation remain unclear. We have previously demonstrated that persistent reflux cholangitis after bilioenterostomy accelerates biliary carcinogenesis through activation of biliary epithelial cell kinetics in hamsters[8-10]. Furthermore, more severe cholangitis is associated with a high occurrence of biliary carcinoma. However, the molecular mechanisms by which chronic biliary inflammation increases the risk of biliary carcinogenesis are obscure, similar to the clinical occurrence in humans. Meanwhile, we have established a method for culturing biliary epithelial cells from the hamster using a collagen gel technique[11].

Chemically reactive oxidants, radicals, and electrophilic mediators, such as hydrogen peroxide and oxyradicals, nitric oxide, malondialdehyde, 4-hydroxynonenal, or eicosanoids, are produced during inflammation, and these chemical mediators are known to induce a variety of biological reactions[2]. Recently, attention has been focused on nitric oxide (NO) as an endogenous mutagen, an angiogenesis factor, and an inhibitor of apoptosis[12]. NO is a free radical that is synthesized from L-arginine by the family of nitric oxide synthases (NOSs). Three

isoforms of the NOSs have been isolated: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS)[13,14]. Although nNOS and eNOS are present constitutively, iNOS is induced in inflamed tissues and generates relatively large amounts of NO, compared with nNOS and eNOS[13-15]. Cytokine and/or lipopolysaccharide stimulation is known to induce iNOS expression in macrophages, hepatocytes, and many other cell types including certain epithelial cells[16-19]. Moreover, iNOS expression and generation of NO in inflamed tissues have been postulated to potentiate epithelial cells to malignant transformation through the ability of NO to promote mutagenic changes in DNA through DNA oxidization and protein nitrosylation[20,21].

In this study, we investigated the role of iNOS activation, NO generation, and DNA damage as the link between chronic inflammation and biliary carcinogenesis, by utilizing normal hamster gallbladder epithelial cells cultured with inflammatory cytokines.

MATERIALS AND METHODS

Animals

Five-week-old female Syrian golden hamsters (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used. The animals were kept under standard laboratory conditions in the Laboratory Animal Center for Biochemical Research at Nagasaki University Graduate School of Biochemical Sciences. All experiments were performed following the Guidelines for Animal Experimentation of Nagasaki University.

Isolation and culture of biliary epithelial cells from hamsters

Biliary epithelial cells were isolated from the biliary tree of hamsters as previously described[11]. After laparotomy, inferior vena cava was paracentesed with a 22G needle, and the liver was perfused in situ with 100 mL Ca^{2+} - and Mg^{2+} - free phosphate-buffered saline (CMF-PBS) containing 10 mmol/L 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) at pH7.4, and 1 mmol/L ethylene glucol-bis (E-aminoethylether) N, N, N', N'-tetraacetic acid (EGTA) for 10 min at 37℃. The vena cava was clamped above the diaphragm, and the perfusing solution was drained *via* the incised portal vein, followed by perfusion with 100 mL Hanks' balanced salt solution (GIBCO, Grand Island, NY) containing 50 mmol/L HEPES and 0.04% collagenase (Nittazeratin, Osaka, Japan) for 10 min at 37℃. After perfusion with the collagenase solution, the liver, gall bladder, and extrahepatic bile duct were removed en bloc. The biliary tree was isolated and separated into the intrahepatic and extrahepatic bile ducts and the gall bladder in CMF-PBS containing 0.1 mmol/L EGTA. The biliary fragments were minced.

The biliary fragments were embedded on collagen gel plated (Collagen Gel Culture kit; Nittazeratin, Osaka, Japan) 60-mm petri dishes with 2 mL of an ice-cold mixture of collagen solution composed of 0.3% acid solution collagen (Cellmatrix Type I -A), 10 × HamF12, and 0.8 N NaOH at 8:1:1 dilution. After incubation at 37℃ with 5% CO_2 and 95% humidity for 20-30 min, collagen gels were overlaid with 5 mL of culture medium composed of Dulbecco's modified Eagle medium/HamF12 medium (DMEM/HamF12, GIBCO) and 10% fetal bovine serum (GIBCO). After incubation of biliary fragments for 7-10 d, the epithelial cells extended widely on the surface of the gel, while the mesenchymal cells progressed toward the inside of the gel. The biliary epithelial cells were isolated from the peripheral region of cellular sheets (Figure 1).

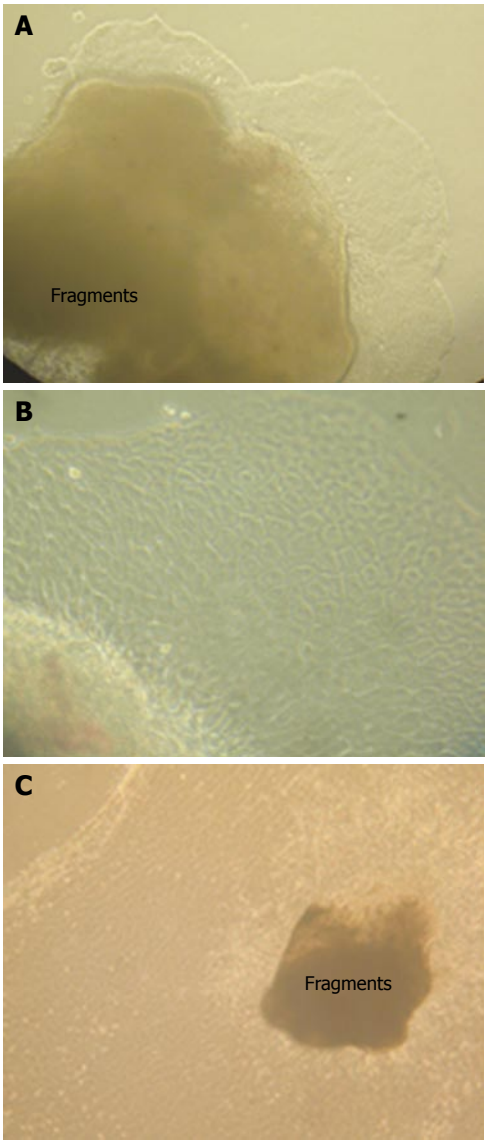

Figure 1 Isolation of hamster biliary epithelial cells. **A**: Phase contrast microscopy revealing a small amount of hamster gallbladder epithelial cells growing on the collagen gels 24 h after culture (× 100); **B**: High magnification of hamster gallbladder epithelial cells demonstrating cuboidal cells around the biliary fragments (× 400); **C**: Phase contrast microscopy showing widely extended gallbladder epithelial cells on the surface of the gel 7 d after culture (× 40).

Addition of inflammatory cytokines

Gallbladder epithelial cells isolated from hamsters were used in this study because of its higher cellular activity compared to other biliary epithelial cells. Resuspended gallbladder epithelial cells (1×10^5 cells/mL) were plated on collagen-coated plates. After incubation for 24 h, the epithelial cells were prepared for three different

Table 1 Oligonucleotide primers used for RT-PCR in this study

Primers	Sequences	References
NOS-590F	GGYTGGTACATGRGCACYGAGATYGG	Cox *et al*[25], 2001
NOS-893R	AAGGCRCARAASTGDGGRTA	Cox *et al*[25], 2001
NOS40F	GCAGGATGGGAAACTGAGGCCCAG	Ramirez-Emiliano *et al*[24], 2005
NOS40R	TGAACAAGGCAGCCAGGTCCCGG	Ramirez-Emiliano *et al*[24], 2005
GAPDHF	TCCCTCAAGATTGTCAGCAA	Liu *et al*[26], 1993
GAPDHR	AGATCCACAACGGATACATT	Liu *et al*[26], 1993

experimental protocols: incubation with culture medium alone (control group), incubation with a cytokine mixture known to increase iNOS expression in other cell types[18,22] consisting of human recombinant interleukin (IL) 1-β (0.5 ng/mL), interferon (IFN)-γ (5 ng/mL), and tumor necrosis factor (TNF)-α (250 ng/mL) (CM group), or incubation with the same cytokine mixture and an iNOS inhibitor L-N (G)-monomethyl arginine (L-NMMA, 0.03 mmol/L) (CM + L-NMMA group). These human recombinant cytokines and L-NMMA were obtained from the Sigma Chemical Co. (St. Louis, MO). Gallbladder epithelial cells in each group were incubated at 37℃ for 24 h, and then processed for the following analyses.

Measurement of NO_2^- and NO_3^- in the medium

To determine the amount of NO produced by gallbladder epithelial cells, nitrite (NO_2^-) and nitrate (NO_3^-) levels were measured in the culture media by high performance liquid chromatography with a NOx analyzer (ENO-10; Eicom, Kyoto)[23].

RT-PCR

iNOS mRNA was amplified using a nested RT-PCR method[24]. The sequences of primers (Invitrogen Life Technologies, Carlsbad, CA) used in this study are shown in Table 1[24-26].

Total RNA was extracted from gallbladder epithelial cells using a RNA extraction kit (ISOGEN; Nippon Gene, Tokyo). Reverse transcription and PCR amplification were performed with a RNA PCR kit Ver.3.0 (Takara Shuzo, Tokyo, Japan). After reverse transcription, the first amplification was performed using 1 μmol/L of the cDNA, together with PCR primers NOS-590F and NOS-893R. The PCR conditions were one cycle of denaturing at 95℃ for 2 min, followed by a touch-down protocol consisting of 18 cycles of 15 s at 95℃, 30 s at 60℃, and 1 min at 72℃, then 25 cycles of 15 s at 95℃, 30 s at 42℃ minus 1℃ per cycle, 1 min at 72℃, and a final extension at 72℃ for 10 min[24]. Next, 1 μL of the cDNA from this amplification was reamplified using the PCR primers specific for iNOS (NOS40F and NOS40R)[24]. The PCR conditions were an initial denaturation at 95℃ for 2 min, followed by a touch-down protocol consisting of 13 cycles of 15 s at 95℃, 30 s at 70℃ minus 1℃ per cycle, 1 min at 72℃, then 30 cycles of 15 s at 95℃, 30 s at 57℃, 1 min at 72℃, and a final extension at 72℃ for 10 min[24]. As a control, amplification of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed[24,26], and the PCR conditions were the same as those described for the first nested PCR amplification. The amplification products were resolved by electrophoresis on 3% (w/v) agarose gels containing 0.1 μg/mL ethidium bromide and photographed under UV trans-illumination.

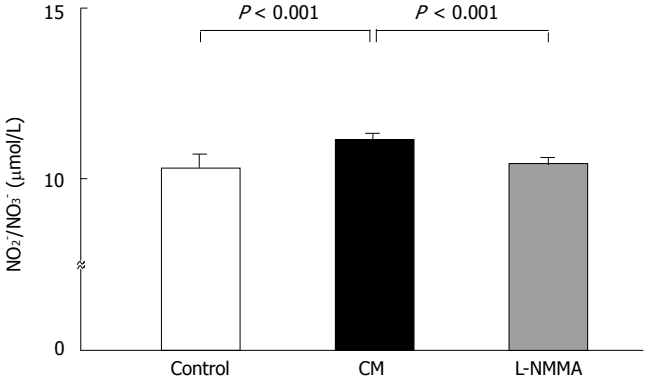

Figure 2 High performance liquid chromatography showing significantly elevated NO_2^- + NO_3^- levels in the CM group compared with the control group, similar NO generation in the CM + L-NMMA group and control group, being significantly lower than that in the CM group.

Comet assay (single cell gel electrophoresis assay)

Comet assay was performed as described previously[27,28]. The gallbladder epithelial cells in each group were resuspended at 1×10^5 cells/mL in ice cold CMF-PBS and combined with molten LMAgarose (Trevigen, Gaitherburg, MD) at a ratio of 1:10 (v/v). The sample was immediately pipetted onto a frosted microscope slide (CometSlide; Trevigen, Gaitherburg, MD). The slides were placed flat at 4℃ in the dark for 10 min, immersed in prechilled lysis solution (Trevigen, Gaitherburg, MD), and left at 4℃ for 30 min to remove cellular proteins, leaving DNA as nucleoids. The slides were then immersed in an alkaline solution (pH > 13, 0.3 mol/L NaOH and 0.001 mol/L EDTA) for 30 min to denature the DNA and hydrolyze sites of damage. The samples were electrophoresed for 10 min and stained with SYBR green I (Trevigen, Gaitherburg, MD) according to the manufacturers instructions. At least 75 cells on each slide, randomly selected by fluorescence microscopy, were analyzed using National Institutes of Health image (Netscape Navigator) with the comet analysis macro (comet 1.4 macro)[29].

Statistical analysis

Values for NO_2^- + NO_3^- levels and proportion of DNA damage were expressed as mean ± SE. For statistical analysis, ANOVA was used to compare non-repeated measurements between groups, followed by Bonferroni correction for paired comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Generation of NO_2^- and NO_3^-

The amount of NO measured by high performance liquid chromatography in each group is shown in Figure 2. The concentration of NO_2^- + NO_3^- in the media was

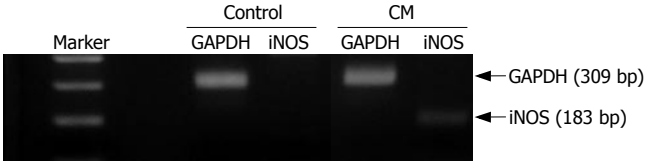

Figure 3 iNOS mRNA expression in gallbladder epithelial cells in the CM group and no iNOS mRNA expression in the control group.

10.35 ± 0.47 μmol/L in the control group (n = 26), 11.06 ± 0.18 μmol/L in the CM group (n = 26), and 10.46 ± 0.18 μmol/L in the CM + L-NMMA group (n = 27). NO generation was significantly higher in the CM group than in the control group ($P < 0.001$). Meanwhile, NO generation in the CM + L-NMMA group and control group was similar, and significantly lower than that in the CM group ($P < 0.001$).

RT-PCR

RT-PCR/touch down amplification of iNOS mRNA in gallbladder epithelial cells is shown in Figure 3. iNOS mRNA (183 bp) expression was demonstrated in the CM, while none of the control cells expressed iNOS mRNA.

Comet assay

Representative fluorescent micrograph images evaluated by single cell gel electrophoresis using comet assay are shown in Figure 4A. In contrast to the intact spherical nuclei observed in the control group, the cells treated with cytokine mixture demonstrated a comet tail indicative of DNA damage. In the CM + L-NMMA group, the cells also exhibited intact spherical nuclei. The ratio of DNA exhibiting comet tails evaluated by using NIH image was 2.14% ± 0.59% in the control group, 5.14% ± 0.69% in the CM group, and 1.70% ± 0.62% in the CM + L-NMMA group (Figure 4B). The level of DNA damage in the CM group was significantly higher than that in the control group ($P < 0.001$), and decreased to the control level after the addition of L-NMMA.

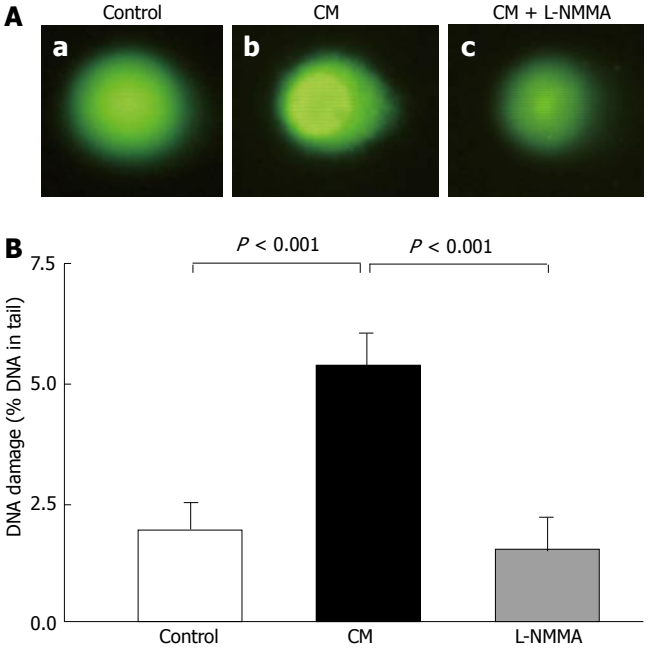

Figure 4 NO-dependent DNA damage evaluated by comet assay. **A**: Fluorescence microscopy showing the apparent comet tail in gallbladder cells stimulated by the cytokine mixture and intact spherical nuclei of the cells in the control group and CM + L-NMMA group; **B**: Analysis of the proportion of DNA migrated to comet tails using NIH image showing a significantly higher level of DNA damage in the CM group than in the control group, which decreased to the control level after the addition of L-NMMA.

DISCUSSION

The induction of iNOS expression and NO production has previously been described in a variety of precancerous or cancerous lesions, in which the NO level of production is in proportion to the degree of malignancy[30,31]. Recently, Jaiswal *et al*[27,32] reported that human cholangiocarcinomas exhibit intensive immunohistochemical staining for iNOS and that cholangiocarcinoma cell lines stimulated by inflammatory cytokines and cholangiocyte cell lines transfected with iNOS produce large amounts of NO due to iNOS expression, which result in both oxidative DNA damage and inhibition of the excision DNA repair process. In the present study, we used primary epithelial cells isolated from the gallbladder because these normal epithelial cells should be suitable for estimating the involvement of iNOS and NO in biliary carcinogenesis, especially in the initiation of biliary carcinoma in response to chronic inflammation.

Following stimulation with a mixture of inflammatory cytokines of IL-1β, IFN-γ, and TNF-α, NO generation was significantly increased in the gallbladder epithelial cells. Furthermore, the production of NO in the presence of inflammatory cytokines was completely suppressed by the addition of an iNOS inhibitor, L-NMMA. Moreover, the expression of iNOS mRNA in the gallbladder epithelial cells was clearly demonstrated in the presence of cytokine-stimulation using nested RT-PCR, but not in the absence of stimulatory cytokines. These findings indicate that inflammatory cytokines promote iNOS expression and NO generation in normal epithelial cells of hamster gallbladder.

NO has the ability to directly oxidize DNA, causing mutagenic changes[20,21]. Although NO also contributes to intracellular communication, inhibition of apoptosis, and enhancement of vascular dilatation, permeability, and neovascularization[33,34], DNA damage may be an essential and initial process involved in the malignant transformation of a wide variety of epithelial cells, and DNA damage in the individual cell can be detected using the highly sensitive comet assay[27,28,32]. In this assay, damaged single- and double-stranded DNA within the nucleus are allowed to migrate toward the anode, by an alkaline hydrolysis process during electrophoresis, resulting in the appearance of a "comet tail". In our study, the comet assay clearly demonstrated that the proportion of DNA moving to the comet tail, i.e., indicating DNA damage, was significantly higher in the cytokine mixture group than in the control group, and that the increased DNA damage was completely inhibited by L-NMMA. These data indicate that inflammatory cytokines stimulate iNOS-mediated DNA damage in normal gallbladder

epithelial cells. Although almost all DNA oxidative breaks can be excised by multiple excision DNA repair processes before mutations occur[35], DNA damage can lead to p53-mediated cell growth arrest and apoptosis, and the accumulation of p53 protein can repress the transcription of iNOS[36]. Furthermore, a recent report has shown that the tumor suppressant organization of normal p53 protein is inhibited in the presence of large amounts of NO in inflamed tissues[37]. In consideration of these facts, the results of our study suggest that NO-mediated oxidative DNA damage produced by inflammatory cytokines through iNOS expression is involved in an initiation process linking chronic biliary inflammation to malignant transformation.

Inflammatory cytokines released in the inflamed tissues initiate the induction of iNOS, resulting in increased NO production. As demonstrated in our study, iNOS-mediated NO production is sufficient to induce DNA damage in normal biliary epithelial cells. In association with persistency of biliary inflammation, the accumulation of NO-mediated genotoxicity may initiate the malignant transformation of epithelial cells lining the biliary tree. Our hamster models of both *in vivo* and *in vitro* tumorigenesis will enhance the understanding of the mechanisms of inflammation-related biliary carcinogenesis.

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COMMENTS

Background

It is well known that biliary carcinoma develops under chronic inflammatory conditions involving the biliary epithelium. We have previously demonstrated that persistent reflux cholangitis after bilioenterostomy accelerates biliary carcinogenesis through activation of biliary epithelial cell kinetics in hamsters. However, the cellular mechanisms of biliary carcinogenesis in response to inflammation remain unclear.

Research frontiers

In the present study, we used primary epithelial cells isolated from the gallbladder of hamsters. These normal epithelial cells should be suitable for estimating the involvement of iNOS and NO in biliary carcinogenesis, especially in initiation of biliary carcinoma in response to chronic inflammation.

Innovations and breakthroughs

In normal hamster gallbladder epithelial cells, cytokine stimulation induced iNOS expression and NO generation which was sufficient to cause DNA damage. The results suggest that NO-mediated oxidative DNA damage produced by inflammatory cytokines through iNOS expression is involved in an initiation process linking chronic biliary inflammation to malignant transformation.

Applications

Our hamster models of both *in vivo* and *in vitro* tumorigenesis will enhance the understanding of the mechanisms of inflammation-related biliary carcinogenesis.

Peer review

The authors investigated the role of inducible nitric oxide (iNOS) in DNA damage using cultured gallbladder epithelial cells. They speculated that the DNA damage would be related to biliary carcinogenesis. The data were interesting.

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BASIC RESEARCH

Intestinal endotoxemia plays a central role in development of hepatopulmonary syndrome in a cirrhotic rat model induced by multiple pathogenic factors

Hui-Ying Zhang, De-Wu Han, Ai-Rong Su, Li-Tong Zhang, Zhong-Fu Zhao, Jing-Quan Ji, Bao-Hong Li, Cheng Ji

Hui-Ying Zhang, Zhong-Fu Zhao, Jing-Quan Ji, Bao-Hong Li, Department of Pathophysiology, Changzhi Medical College, Changzhi 046000, Shanxi Province, China
De-Wu Han, Institute of Hepatology, Shanxi Medical University, Taiyuan 030001, Shanxi Province, China
Ai-Rong Su, Department of Physiology, Bengbu Medical College, Bengbu 233030, Anhui Province, China
Li-Tong Zhang, Department of Gynaecology, Shanxi Modern Women Hospital, Taiyuan 030012, Shanxi Province, China
Cheng Ji, Department of Medicine, University of Southern California, Los Angeles, CA 90033, United States
Supported by the Awards to University Academic Leaders Granted by the Government of Shanxi Province of China to Hui-Ying Zhang and partially by the US National Institute of Health, NIAAA, Grant R01 AA014428 to Cheng Ji
Correspondence to: Hui-Ying Zhang, MD, Professor, Department of Pathophysiology, Changzhi Medical College, 161 Jie Fang Dong Jie, Changzhi 046000, Shanxi Province, China. zhhy2001@hotmail.com
Telephone: +86-355-3031235 Fax: +86-355-3034065
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Abstract

AIM: To characterize the correlation between severity of hepatopulmonary syndrome (HPS) and degree of hepatic dysfunction, and to explore how intestinal endotoxemia (IETM) affects the development of HPS in cirrhotic rats.

METHODS: Male Wister rats were fed with a diet containing maize flour, lard, cholesterol, and alcohol and injected subcutaneously with CCl_4 oil solution every two days for 8 wk to induce typical cirrhosis and development of HPS. The animals were also given a nitric oxide (NO) production inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) intraperitoneally, and an iNOS inhibitor, aminoguanidine hydrochloride (AG) via gavage daily from the end of the 4th wk to the end of the 6th or 8th wk, or a HO-1 inhibitor, zinc protoporphyrin (ZnPP) intraperitoneally 12 h prior to killing. Blood, liver and lung tissues were sampled.

RESULTS: Histological deterioration of the lung paralleled to that of the liver in the cirrhotic rats. The number of pulmonary capillaries was progressively increased from 6.1 ± 1.1 (count/filed) at the 4th wk to 14.5 ± 2.4 (count/filed) at the 8th wk in the cirrhotic rats. Increased pulmonary capillaries were associated with increased blood levels of lipopolysaccharide (LPS) (0.31 ± 0.08 EU/mL *vs* control 0.09 ± 0.03 EU/mL), alanine transferase (ALT, 219.1 ± 17.4 U/L *vs* control 5.9 ± 2.2 U/L) and portal vein pressure. Compared with normal control animals, the number of total cells in bronchoalveolar lavage fluid (BALF) of the cirrhotic rats at the 8th wk was not changed, but the number of macrophages and the ratio of macrophages to total cells were increased by nearly 2-fold, protein expression of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) started to increase significantly at the 4th wk, and reached its peak at the 8th wk in the lung of cirrhotic rats. The increase of iNOS expression appeared to be quicker than that of eNOS. NO_2^-/NO_3^- was also increased, which was correlated to the increase of iNOS (r = 0.7699, P < 0.0001) and eNOS (r = 0.5829, P < 0.002). mRNA expression of eNOS and iNOS was highly consistent with their protein expression.

CONCLUSION: Progression and severity of HPS as indicated by both increased pulmonary capillaries and histological changes are closely associated with LPS levels and progression of hepatic dysfunction as indicated by increased levels of ALT and portal vein pressure. Intestinal endotoxemia plays a central role in the development of HPS in the cirrhotic rat model by inducing NO and/or CO.

Key words: Endotoxin; Alcohol; Nitric oxide synthase; Hemeoxygenase-1; Capillary; Macrophage; Cirrhosis; Hepatopulmonary syndrome

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INTRODUCTION

Hepatopulmonary syndrome (HPS) develops when arterial oxygenation deficiency occurs due to intrapulmonary vascular dilatations that are often associated with severe hepatic disease[1]. Recent studies support that the presence

of HPS significantly increases the mortality of cirrhotic patients, particularly those with decompensated liver disease[2,3].

The pathogenesis and precise mechanism of pulmonary vascular abnormalities in HPS are the fields of active investigation. So far, studies have been focused on characterizing the increased pulmonary production of vasodilator substances, most notably nitric oxide (NO). Pulmonary NO overproduction in human HPS, as assessed by exhaled NO levels, has also been reported[4,5]. It was reported that HPS patients showed short term improvements in oxygenation after acute administration of L-NAME (a specific nitric oxide synthase inhibitor) or methylene blue (an inhibitor of cyclic guanosine monophosphate generation)[6-8], indicating that pulmonary NO and cyclic guanosine monophosphate production can lead to changes in pulmonary vascular tone, thus affecting oxygenation. However, the role of pulmonary NO in lung oxygenation is still controversial. Inhalation of L-NAME could not acutely improve intrapulmonary vasodilatation[9], and sequential inhibition of the nitric oxide-cyclic guanosine monophosphate pathway by curcumin (diferuloylmethane), terlipressin and methylene blue could not improve the intrapulmonary shunt, and both hypoxaemia and orthodeoxia were substantially worsened after treatment with the above three durgs[10], strongly suggesting that factors other than nitric oxide synthase (NOS)-derived NO effects on vascular tone contribute to HPS. It has also been shown that the levels of COHb indicative of CO production are increased in cirrhotic patients who develop HPS compared to the cirrhotic patients who do not develop HPS and correlate with gas exchange abnormalities, suggesting that CO may also contribute to human HPS[11].

The sequelae of endotoxemia are often fatal. The lung appears to be an organ sensitive to endotoxemia and many distant-focus infections can be detected as a result of pulmonary dysfunction[12,13]. Patients with hepatic cirrhosis have an elevated plasma level of lipopolysacchride (LPS)[14], which is a leading causative factor for cirrhotic complications, such as hepatorenal syndrome[15-18] and hepatic encephalopathy[19].

We have previously developed a rat model characteristic of cirrhosis and HPS that can be non-invasively induced by multiple pathogenic factors including high fat diet, alcohol, cholesterol, corn flour and CCl_4[20]. A significantly increased level of LPS in plasma is closely related to the deceased blood oxygen content and intrapulmonary vascular dilatation in the rat model, suggesting that intestinal endotoxemia (IETM) and its accompanying cytokines, such as TNF-α, play a role in the pathogenesis of HPS.

This study was to further characterize the relation between severity of HPS and degree of hepatic dysfunction, and to explore how IETM affects the development of HPS in cirrhotic rats. The results show that the severity of HPS parallels to the levels of blood LPS and progression of hepatic dysfunction and IETM plays a central role in the development of HPS in cirrhotic rats.

MATERIALS AND METHODS

Animals and reagents

Male Wister rats, weighing 220-250 g, were obtained from the Animal Center of Shanxi Medical University. All animals received human care during the study. The study was approved by the Ethical and Research Committee of the Shanxi Medical University. Experiments were performed following the institutional guideline for animal research. Cirrhosis and HPS were induced in the rats as previously described[20]. In brief, male Wister rats were fed with a complex diet containing maize flour, lard, cholesterol, and alcohol for 8 wk, and injected subcutaneously with CCl_4 oil solution every two days over the experimental period to induce typical cirrhosis and development of HPS. The animals were also given a NO production inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) intraperitoneally, 5 mg/kg per day, or an inducible nitric oxide synthase (iNOS) inhibitor, aminoguanidine hydrochloride (AG) *via* gavage, 100 mg/kg per day from the end of the 4th wk to the end of the 6th or 8th wk, or 50 μmol/kg hemeoxygenase-1 (HO-1) inhibitor, zinc protoporphyrin (ZnPP) intraperitoneally 12 h prior to killing. The animals were randomized into four groups: (1) normal control group (fed with standard diet), (2) 4-wk group (fed with the complex diet for 4 wk), (3) 6-wk group (fed with the complex diet for 6 wk), and (4) 8-wk group (fed with the complex diet for 8 wk). Blood, liver and lung tissues were sampled from the rats.

Limulus amebocyte lysate (LAL) reagent, for determination of LPS in plasma, was provided by Shanghai Yi Hua Scientific, Inc (Shanghai, China). Tumor necrosis factor α (TNF-α) radioimmunoassay kit was provided by Radioimmunity Institute of PLA General Hospital (Beijing, China). Detecting kit for NO_2^-/NO_3^- was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Polyclone anti- endothelial nitric oxide synthase (eNOS) and anti- inducible nitric oxide synthase (iNOS) were purchased from Beijing Zhongshan Biotic Reagent Company (Beijing, China). Immunohistochemistry kit for hemeoxygenase-1 (HO-1) was obtained from Wuhan Boshide Bioengeering Institue (Wuhan, China). TRIzol reagent was bought from the Life Technologies Company (California, USA). Reverse transcriptase polymerase chain reaction (RT-PCR) primers for β-actin, eNOS, and iNOS were synthesized by Shanghai Jiebeist Gene Company, LTD (Shanghai, China). NO inhibitor L-NAME, specific iNOS inhibitor AG, and HO-1 inhibitor ZnPP were obtained from Sigma Chemical Co (St. Louis, MO).

Measurement of LPS, TNF-α, alanine transferase, malondialdehyde *in plasma, NO_2^-/NO_3^- and COHb in pulmonary homogenate and portal vein pressure*

The contents of LPS, TNF-α, alanine transferase (ALT), malondialdehyde (MDA) in plasma, NO_2^-/NO_3^- (indirectly reflecting NO) and COHb (indirectly reflecting CO) in pulmonary homogenate were measured with the different kits following their manufacturers'instructions. The method described by MacPhail *et al*[21] was modified for

measurement of portal vein pressure (PVP) in which 18-gauge over-the-needle catheter was inserted into portal vein and connected to a water manometer.

Histology and calculation of capillaries

Samples from the liver and lung were collected and fixed immediately in 100 g/L phosphate-buffered formaldehyde overnight. After paraffin embedding, serial 4-μm thick sections were prepared and stained with hematoxylin and eosin (HE). Images (× 100) of liver and lung sections were captured with Olympus microscope equipped with a digital camera. The number of capillaries was counted in 6 fields per slide per group (n = 6) with the Image-Pro plus full automatism analysis system.

Number of cells in bronchoalveolar lavage fluid

Bronchoalveolar lavage was performed with 0.9% sterile NaCl. The number of total cells and macrophages in bronchoalveolar lavage fluid (BALF) was counted respectively with a hemocytometer and the ratio of macrophages to total cells was calculated.

Dynamic study of eNOS, iNOS and HO-1 protein expression in lung architecture

Samples from the right lobe of lung were collected into 100 g/L phosphate-buffered formaldehyde and fixed overnight. Serial 4-μm thick sections were prepared after the samples were dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in paraplast. Expression of eNOS, iNOS and HO-1 was analyzed with immunohistochemical staining and quantified with the Image-Pro plus full automatism analysis system in a blind fashion.

Dynamic study of eNOS and iNOS mRNA expression in lung

RT-PCR was employed to detect the mRNA expression of eNOS and iNOS genes in lung. The primer sequences used are as follows: eNOS (435 bp)[22]: 5'-CTGCTGCCCG AGATATCTTC-3', 5'-AAGTAAGTGAGAGCCTGGCG CA-3'; iNOS (388 bp)[23]: 5'-AGCATCACCCCTGTGTTC CACCC-3', 5'-TGGGGCAGTCTCCATTGCCA-3'; β-actin (630 bp, as control)[24]: 5'-GATGGTGGGTAT GGGTCAGAAGGAC-3', 5'-GCTCATTGCCGATAG TGA TGA CT-3'.

Total RNA was extracted from 50-100 mg snap-frozen left lung tissue following TRIzol protocol. Following precipitation, the RNA was resuspended in RNase-free buffer, the concentration was determined by measuring the ultra-violet light absorbance at 260 nm and 280 nm and the purity of RNA was estimated from the ratio of A_{260}/A_{280}.

Single-stranded complementary DNA (cDNA) was synthesized from the total RNA. Two μg RNA was mixed with 0.5 μg oligo (dT), pre-incubated in 15 μL of diethylpyrocarbonate (DEPC) at 70℃ for 5 min, and rapidly chilled on ice. Five μL of M-MLV 5 × reaction buffer, 1.25 μL of dNTP (10 mmol/L, each), 25 units of rRNasin ribonuclease inhibitor, 200 units of M-MLV RT and DEPC-treated water were added into the annealed primer/template to a final volume of 25 μL. The reaction solution was incubated at 42℃ for 60 min and terminated by placing it on ice after denaturation at 85℃ for 5 min. The resulting cDNA was used as a template for subsequent PCR.

The PCR mixture contained 5 μL of 10 × Taq buffer, 1 μL of dNTP (10 mmol/L, each), 1 μL of gene specific primers, 2.0 units of Taq DNA polymerase and 1 μL of cDNA in a total volume of 50 μL. Thirty-five cycles of amplification were performed with an initial incubation at 94℃ for 3 min and a final extension at 72℃ for 7 min, each cycle consisted of denaturation at 94℃ for 1 min, annealing at 64℃ for 1 min and extension at 72℃ for 1 min. The quantities of cDNA producing equal amounts of β-actin-PCR-product were used in PCR with the primers for iNOS and eNOS. Following RT-PCR, 10 μL samples of amplified products was resolved by electrophoresis on 1.2% agarose gel and stained with ethidium bromide. The level of each PCR product was semi-quantitatively evaluated using a digital camera and an image analysis system (Alpha Innotech Co., USA), and normalized to β-actin.

Statistical analysis

Data were evaluated by analysis of variance, multiple comparisons between groups were performed with SPSS software. All values are reported as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of histological features of both liver and lung

Pulmonary pathological changes were consistent with hepatic pathological changes towards the same direction, namely, pulmonary pathological changes became progressively exacerbated as liver injury progressed. In normal liver architecture, sinusoids and cord located in order around central veins (Figure 1A). Derangement of hepatic cord and central lobular necrosis with infiltration of inflammatory cells, cytoplasm rarefaction in liver cells with balloon-like alteration and steatosis, and increased fiber amount in interlobular area were found in the 4th wk group (Figure 1B). Furthermore, more typical balloon-like alteration and steatosis, fiber network formation, further aggravated infiltration of inflammatory cells were observed in the 6th wk group (Figure 1C). False lobule formation, secondary degeneration and necrosis in liver cells with infiltration of inflammatory cells were demonstrated in the 8th wk group (Figure 1D). Thin alveoli wall with disseminated pulmonary phagocytes was observed in the normal control group (Figure 2A). Thick alveoli wall with infiltration of phagocytes and neutrophils was observed in the 4th wk group (Figure 2B). Dilated capillaries and increased capillary density in further wider septum, and partially decreased air space in their size with infiltration of phagocytes and neutrophils were found in the 6th wk group (Figure 2C). Obviously dilated capillaries and increased number of capillaries, massive enlarged phagocytes within wider septa and air spaces with focal narrower air spaces were found in the 8th wk group (Figure 2D).

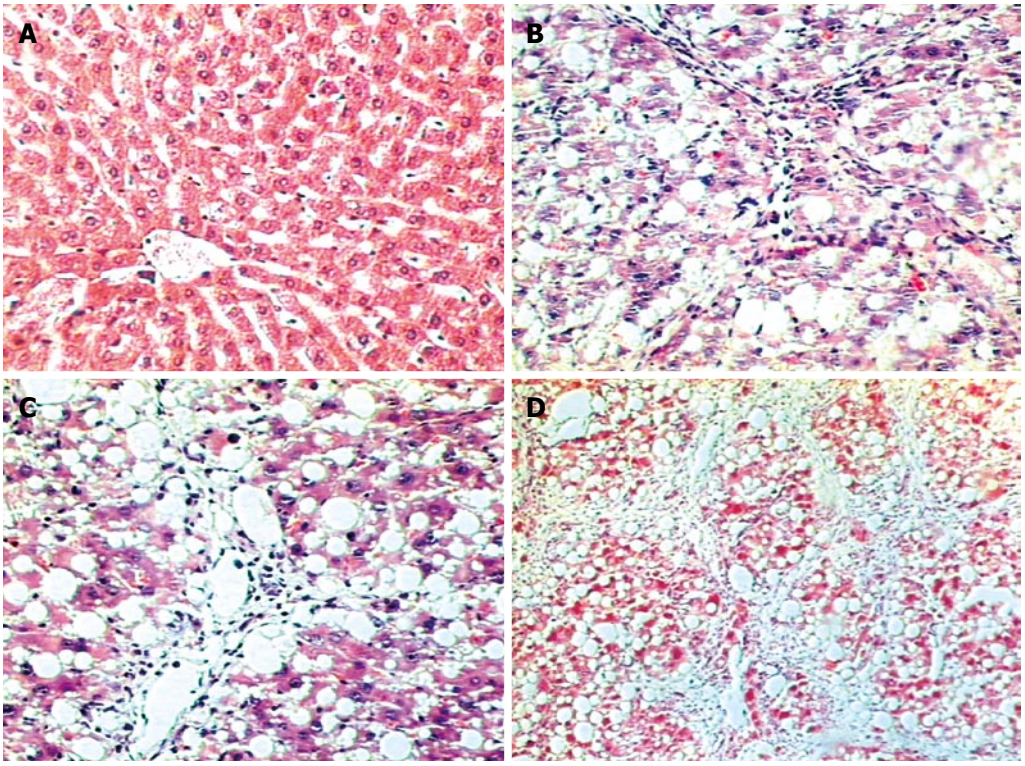

Figure 1 Liver histology of HPS (HE, × 100) in normal control group (**A**), 4th wk group (**B**), 6th wk group (**C**) and 8th wk group (**D**).

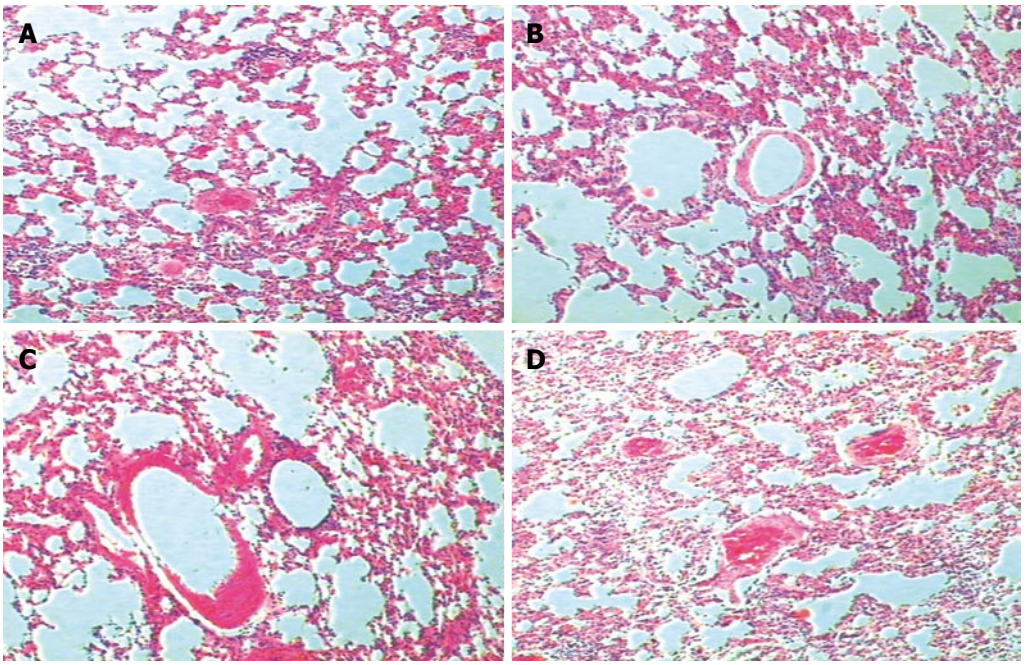

Figure 2 Lung histology of HPS (HE, × 200) in normal control group (**A**), 4th wk group (**B**), 6th wk group (**C**) and 8th wk group (**D**).

Changes in pathological parameters

The levels of LPS, TNF-α, ALT and MDA in plasma were progressively increased over the period of 8 wk. PVP was significantly elevated in the 4th wk and persisted up to the 8th wk (Table 1).

We have previously reported that the pulmonary capillaries are dilated in the lung of cirrhotic rats based on Tc99MAA labeling and scanning[20]. To know if proliferation of capillaries also occurs during the development of HPS, we counted the number of capillaries. The number of pulmonary capillaries was progressively increased in cirrhotic rats (Figure 3), which was positively related to the increased levels of LPS, TNF-α, ALT and MDA in plasma as well as to the NO and CO in pulmonary homogenate (Table 2).

The number of cells in bronchoalveolar lavage fluids could reflect the function of lung. Compared with normal control animals, the total number of cells was not changed. However, the number of macrophages and the ratio of macrophages to total number of cells were significantly increased in BALF of cirrhotic rats at the 8th wk, strongly suggesting that macrophages could play a role in the pathogenesis of HPS (Table 3).

Table 1 Levels of endotoxin, TNF-α, ALT and MDA in plasma and PVP (mean ± SD, *n* = 6)

Groups	Endotoxin (Eu/mL)	TNF-α (ng/mL)	ALT (u/L)	MDA (nmol/mL)	PVP (H_2O cm)
Normal	0.09 ± 0.03	0.53 ± 0.16	5.85 ± 2.24	0.06 ± 0.03	10.31 ± 1.16
4th wk	0.19 ± 0.03[a]	1.37 ± 0.35[a]	56.24 ± 14.73[a]	0.10 ± 0.05	17.54 ± 1.84[a]
6th wk	0.24 ± 0.04[a]	1.68 ± 0.23[a]	53.50 ± 22.10[a]	0.18 ± 0.03[a,c]	15.44 ± 3.11[a,c]
8th wk	0.31 ± 0.08[a,e]	2.42 ± 0.38[a,e]	219.10 ± 17.37[a,e]	0.26 ± 0.06[a,e]	18.38 ± 2.53[a,e]

[a]$P < 0.05$ *vs* normal group; [c]$P < 0.05$ *vs* 4th wk group; [e]$P < 0.05$ *vs* 6th wk group.

Table 2 Correlation analysis (*n* = 6)

Groups	*r*	*P*
LPS *vs* quantities of pulmonary capillaries	0.626	< 0.001
TNF-α *vs* quantities of pulmonary capillaries	0.644	< 0.001
ALT *vs* quantities of pulmonary capillaries	0.556	< 0.01
MDA *vs* quantities of pulmonary capillaries	0.691	< 0.0002
CO *vs* quantities of pulmonary capillaries	0.432	< 0.01
NO *vs* quantities of pulmonary capillaries	0.725	< 0.0001

Table 3 Changes of cell numbers in bronchoalveolar lavage fluid (mean ± SD, *n* = 6)

Groups	Total cell numbers (/μL)	Macrophages (/μL)	Ratio of macrophage/ total cell numbers
Normal control	582.7 ± 74.5	184.1 ± 5.4	0.32 ± 0.05
8th wk	619.2 ± 116.5	310.8 ± 27.5[a]	0.51 ± 0.06[a]

[a]$P < 0.05$ *vs* normal control group.

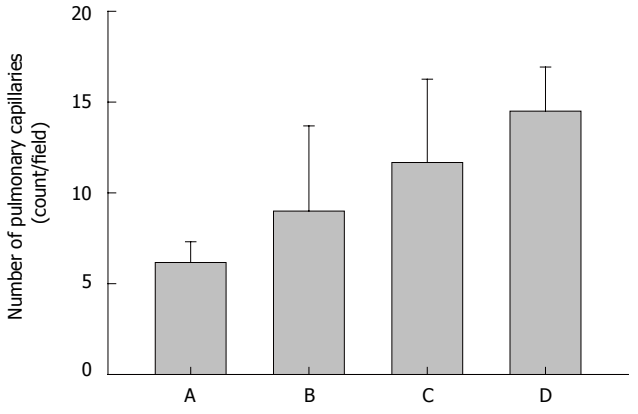

Figure 3 Change in the number of pulmonary capillaries in normal control group (A), 4th wk group (B), 6th wk group (C) and 8th wk group (D). [a]$P < 0.05$ B, C, D *vs* A; [a]$P < 0.05$ D *vs* B.

Levels of NO_2^-/NO_3^-, eNOS and iNOS in lung

Immunohistochemistry displayed that eNOS protein expression in pulmonary architecture started to increase significantly at the 4th wk and reached its peak at the 8th wk (Table 4). The change in iNOS protein expression was similar to that of eNOS, but significant differences were detected between the 4th and 6th wk as well as between the 6th and 8th wk, showing a progressive ascending. NO_2^-/NO_3^- was nearly increased by 3.5-fold at the 4th wk, by 5-fold at the 6th wk, and by more than 7-fold at the 8th wk.

Table 4 Levels of NO2-/NO3-, expression of eNOS and iNOS in lung (mean ± SD, *n* = 6)

Groups	NO_2^-/NO_3^- (μmol/g protein)	eNOS (IOD)	iNOS (IOD)
Normal control	3.6 ± 1.7	2385.6 ± 752.4	4214.8 ± 1783.7
4th wk	12.6 ± 3.7[a]	6448.9 ± 922.7[a]	8498.7 ± 2703.7[a]
6th wk	18.9 ± 5.5[a,c]	8008.9 ± 2812.7[a]	12831.6 ± 4901.2[a,c]
8th wk	26.9 ± 6.4[a,e]	14704.7 ± 5779.9[a,e]	19173.4 ± 2401.2[a,e]

[a]$P < 0.05$ *vs* normal group; [c]$P < 0.05$ *vs* 4th wk group; [e]$P < 0.05$ *vs* 6th wk group.

Table 5 Levels of eNOS and iNOS mRNA expression in lung (mean ± SD, *n* = 6)

Groups	eNOS	iNOS
Normal control	0.62 ± 0.07	0.22 ± 0.01
4th wk	0.65 ± 0.08[a]	0.98 ± 0.03[a]
6th wk	0.68 ± 0.07[a]	1.23 ± 0.03[a,c]
8th wk	0.75 ± 0.08[a,e]	1.46 ± 0.05[a,e]

[a]$P < 0.05$ *vs* normal group; [c]$P < 0.05$ *vs* 4th wk group; [e]$P < 0.05$ *vs* 6th wk group.

The increased NO_2^-/NO_3^- was more relevant to the expression of iNOS ($r = 0.7699$; $P < 0.01$) than to that of eNOS ($r = 0.5829$, $P < 0.002$).

The expression of eNOS and iNOS was distributed differently in the lung tissue stained with immunohistochemistry. In normal lung, eNOS was expressed in pulmonary capillary endothelium. In contrast, eNOS was expressed not only in the endothelium but also in type Ⅰ and Ⅱ epithelial cells and macrophages, and the expression was progressively increased in the lung of cirrhotic rats over the experimental period (Figure 4). iNOS was located mainly in macrophages and type Ⅱ epithelial cells during the development of HPS.

At mRNA levels, the expression of eNOS and iNOS in the lung was highly consistent with their protein expression (Table 5, Figure 5). Plasma endotoxin was significantly correlated with the expression of eNOS and iNOS over the 8 wk period (Table 6), suggesting that IETM might induce the expression of eNOS and/or iNOS in the development of HPS.

Effects of NOS or HO-1 inhibitors on expression of HO-1 or NOS in lung

The level of COHb and expression of HO-1 in the lung of cirrhotic rats began to increase at the 4th wk and reached their peak at the 8th wk (Table 7). The location of

Table 6 Analysis of correlation (n = 6)

Pairs	r	P value
Plasma endotoxin *vs* eNOS protein	0.6348	$P < 0.01$
Plasma endotoxin *vs* eNOS mRNA	0.7490	$P < 0.01$
Plasma endotoxin *vs* iNOS protein	0.5710	$P < 0.01$
Plasma endotoxin *vs* iNOS mRNA	0.8980	$P < 0.01$
Plasma TNF-α *vs* eNOS protein	0.8354	$P < 0.01$
Plasma TNF-α *vs* iNOS protein	0.8538	$P < 0.01$

Table 7 Changes of COHb and HO-1 protein in lung (mean ± SD, n = 6)

Groups	COHb (mg/g protein)	HO-1 protein (IOD)
Normal Control	0.13 ± 0.06	1515.18 ± 981.50
4th wk	0.27 ± 0.06[a]	7391.47 ± 674.97[a]
6th wk	0.29 ± 0.06[a]	11 157.03 ± 6093.39[a]
8th wk	0.43 ± 0.98[a,e]	24 867.59 ± 11 054.95[a,e]

[a]$P < 0.05$ *vs* normal group; [e]$P < 0.05$ *vs* 6th wk group.

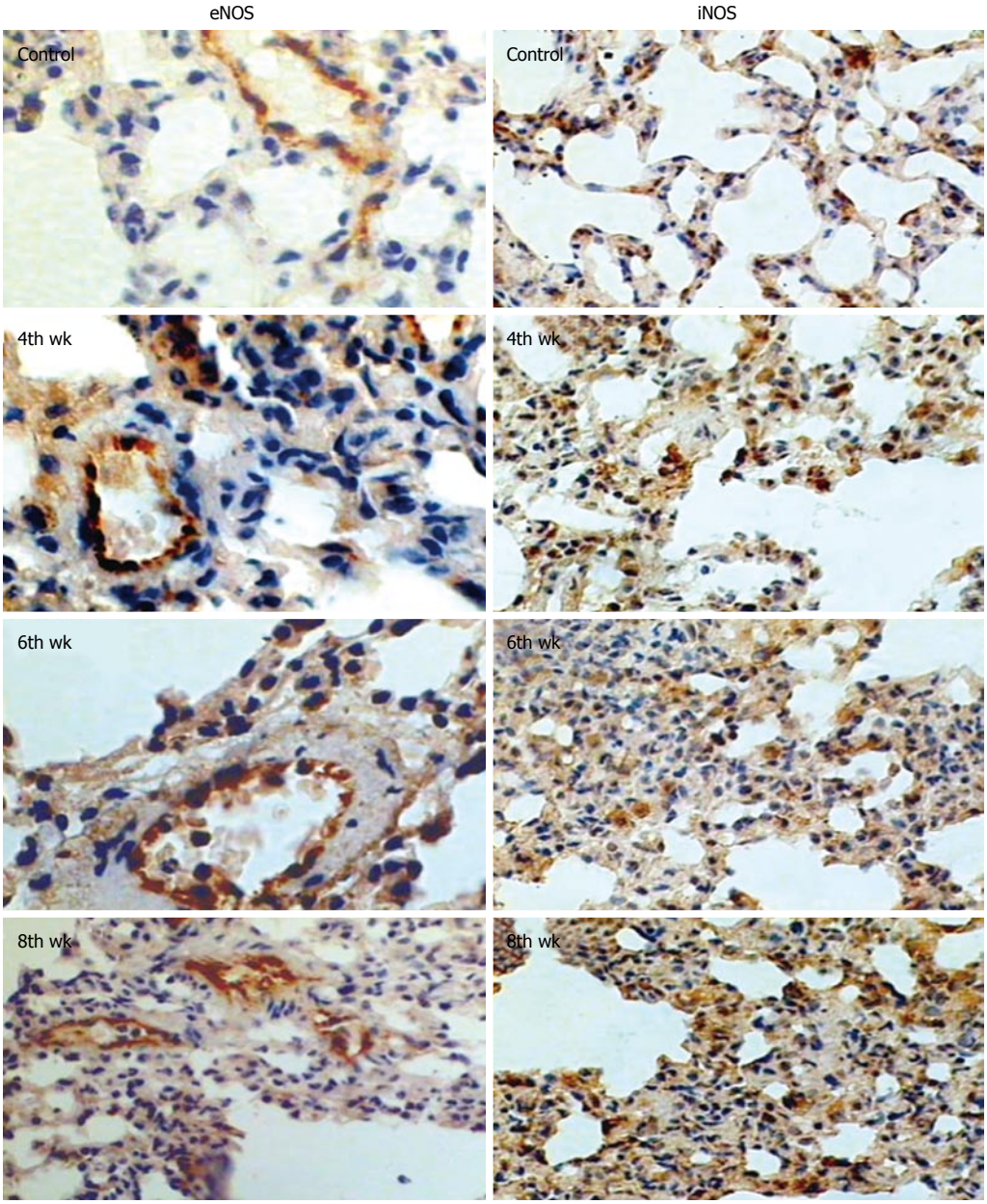

Figure 4 Immunohistochemistry of NOS expressions in the lung of rats with their eNOS and iNOS stained brown (× 200).

HO-1 was mainly found in pulmonary type Ⅱ epithelial cells, macrophages, and capillary endothelium (Figure 6). The levels of plasma LPS and TNF-α were closely correlated with COHb and expression of HO-1 (Table 8).

The expression of HO-1 in cirrhotic rats was decreased by 3-fold at the 6th wk when the non-specific inhibitor (L-NAME) of NOS was administrated and by 5-fold when the specific inhibitor (AG) of iNOS was administrated (Table 9). Similar effects were also detected at the 8th wk. Inhibition of HO-1 expression specifically by ZnPP in cirrhotic rats decreased significantly the expression of eNOS and iNOS (Table 10), indicating that both CO and NO were involved in the development of HPS.

DISCUSSION

In this study, dynamic alterations in plasma endotoxin were found to be closely associated with the expression

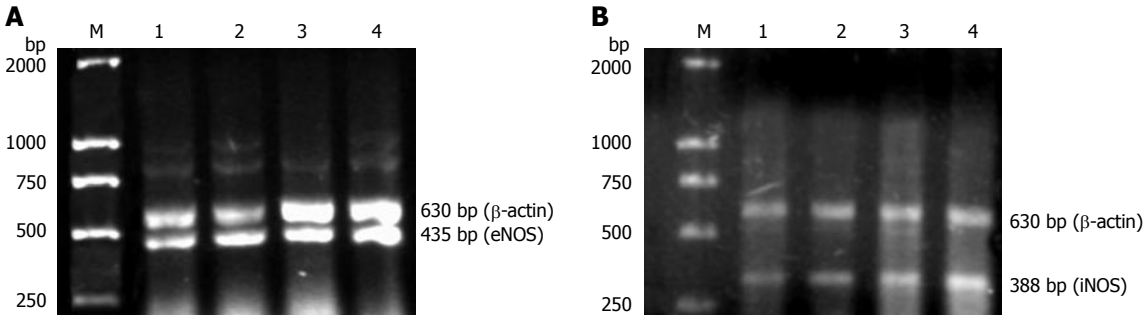

Figure 5 Expression of eNOS mRNA (**A**) and iNOS mRNA (**B**) in rat lung. M: DNA size marker; 1: control; 2: 4th wk group; 3: 6th wk group; 4: 8th wk group.

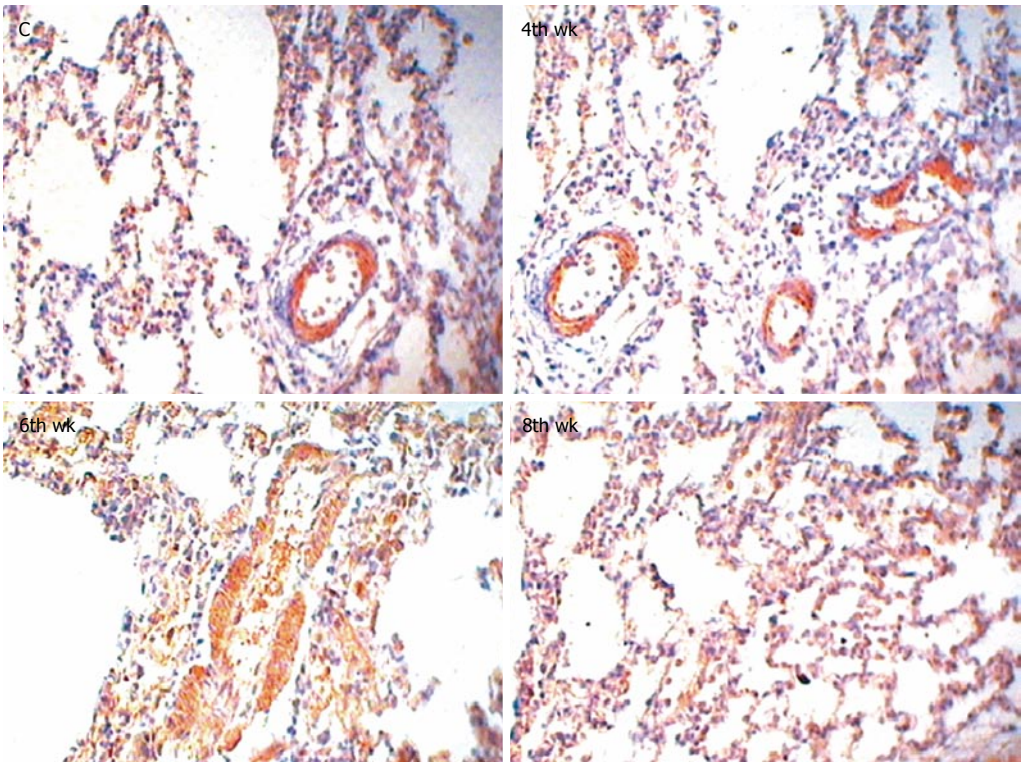

Figure 6 Immunohistochemistry of HO-1 expressions in lung of rats with HO-1 stained brown (× 200).

Table 8 Correlation analysis (n = 6)

Groups	r	P
LPS *vs* COHb	0.876	< 0.01
LPS *vs* HO-1	0.802	< 0.01
TNF-α *vs* COHb	0.755	< 0.01
TNF-α *vs* HO-1	0.796	< 0.05

Table 9 Effect of L-NAME and AG on HO-1 expression (mean ± SD, n = 6; IOD)

Groups	6th wk	8th wk
Cirrhotic control	11157.03 ± 6093.39	24867.59 ± 11054.95
L-NAME + cirrhosis	3455.13 ± 1370.03[a]	11063.48 ± 426.48[a]
AG + cirrhosis	2286.59 ± 6181.20[a]	9828.32 ± 799.51[a]

[a]P < 0.05 *vs* cirrhotic control group.

Table 10 Specific effect of ZnPP on expression of eNOS and iNOS (mean ± SD, n = 6)

Groups	eNOS (IOD)	iNOS (IOD)
6th wk cirrhotic control	13206.7 ± 8490.9	9401.1 ± 691.9
6th wk ZnPP	2025.7 ± 6097.1[a]	3500.3 ± 237.4[a]
8th wk cirrhotic control	21198.1 ± 11865.3	26419.8 ± 3560.5
8th wk ZnPP	2406.3 ± 9117.9[a]	3522.8 ± 356.5[a]

[a]P < 0.05 *vs* cirrhotic control group.

of eNOS, iNOS, HO-1, the number of capillaries, the histological changes characteristic of HPS in cirrhotic rats, demonstrating that the function of lung and liver is simultaneously deteriorated, both CO and NO are involved in pulmonary vascular abnormalities, pulmonary macrophages play a role in HPS of hepatic cirrhosis rats.

Whether the presence and severity of HPS are associated with the severity of underlying liver disease remains unclear. It was reported that HPS occurs more common in advanced cirrhosis[25-29]. HPS can also occur in acute[30] and chronic noncirrhotic hepatitis[31,32]. In patients with hypoxic hepatitis, intrapulmonary vasodilatation indicative of HPS frequently occurs and can be reversed

after normalization of hepatic dysfunction[33], indicating that HPS is interrelated with underlying liver disease. In contrast, other studies reported that HPS occurs both in extrahepatic portal venous obstruction[34] and in hepatic venous outflow obstruction without hepatic cirrhosis[35], suggesting that severe liver dysfunction and cirrhosis are not absolutely associated with the development of HPS. In the present study, the characteristic histological changes in both liver and lung during the development of HPS were observed in cirrhotic rats, indicating that there is a paralleling deterioration of lung and liver function, which supports our hypothesis that hepatic pathological alteration is a substantial basis for the development of HPS in cirrhotic rats.

Oxygenation of red blood cells occurs in the alveolar-capillary network where the pulmonary capillary bed and alveoli come together in the alveolar wall achieving optimal gas exchange. Because the diameter of pulmonary capillaries (about 6 μm) is slightly smaller than that of red blood cells (8 μm), red blood cells must flow through in a single file and change their shape as they pass through the pulmonary capillaries. This shape change ensures a minimal distance between oxygen in alveoli and hemoglobin in red cells. Passive changes in the diameters of pulmonary capillaries are caused by alterations in pre- and post-capillary resistance. Injection of micro-opaque gelatin into pulmonary arteries of autopsy specimens from patients with cirrhosis has documented the presence of pre-capillary pulmonary vascular dilatations and direct arteriovenous communications[36]. The resulting consequence is the distending of pulmonary capillaries with increased blood leading to incomplete penetration of oxygen through dilated vessels that abut upon alveoli and true anatomic shunts in which deoxygenated blood is directly shunted into arterial blood bypassing gas exchange units. Therefore, it is believed that hypoxemia occurs as a result of one (or the combination of several) of the following mechanisms: (1) ventilation-perfusion mismatching (reflecting excess perfusion for a given ventilation), (2) diffusion-perfusion impairment (due to an increased oxygen diffusion distance from alveoli to hemoglobin across the dilated vessels), and (3) true intrapulmonary anatomical shunts[37-42]. It was reported that vascular abnormalities in HPS are associated with the increased number of dilated pre-capillaries, capillaries and pre-capillary arteriovenous communications[43]. In experimental HPS, the increased number of pulmonary vascular capillaries can be observed under electron microscope[44], but no detailed analysis is available. Lung perfusion imaging with Tc99MAA can show broadened pulmonary capillaries in rats with HPS[20]. Furthermore, in the present study, a number of progressively increased pulmonary capillaries were found as cirrhosis progresses in rats, confirming the probability of remodeling, angiogenesis or vasculogenesis proposed by Gomez *et al*[9]. The increased number of pulmonary capillaries is positively correlated with both dynamically increased levels of LPS, TNF-α, and MDA in plasma, further demonstrating that LPS plays a central role in the development of HPS in cirrhotic rats[20]. The increased number of pulmonary capillaries is also positively correlated with dynamically increased ALT. Moreover, a clinical syndrome similar to HPS has been observed in congenital disorders without liver injury in which either hepatic venous blood flow does not reach the lung [45] or portal venous blood reaches the inferior vena cava without passing through the liver[46], indicating that factors either produced or metabolized in the liver can modulate the pulmonary vasculature and play a key role in maintaining the normal pulmonary vascular integrity. In addition, liver transplantation can reverse the ventilation-perfusion mismatch seen in HPS and restore normoxaemia[47]. These findings further support our notion that hepatic pathological alteration is a substantial basis for the development of HPS.

The role of NO in cirrhotic hyperdynamic circulation and induction of iNOS in endothelial cells by cytokines and endotoxin has been suggested as the mechanism[48], which soon appears as a very likely candidate for HPS[49]. However, the involvement of NOS isoform in increased NO production in the lung is controversial[50-53]. In the present study, the expression of eNOS and iNOS started to increase at the 4th wk and was closely related to the increased plasma LPS. Interestingly, the expression of iNOS not eNOS was significantly increased at the 6th wk compared to that at the 4th wk and the increased levels of nitric and nitrate in pulmonary homogenate exhibited a more significant linear correlation with iNOS than with eNOS, suggesting that iNOS may be more important in the development of HPS. In addition, a significantly increased quantity of macrophages in bronchoalveolar lavage fluid was observed during HPS in cirrhotic rats. These results demonstrate that the elevated NO results preferably from iNOS of pulmonary macrophages and, to a lesser extent, from eNOS in the lung of cirrhotic rats. Pulmonary macrophages may be a leading contributor to the development of HPS. Meanwhile, COHb in homogenate and HO-1 expression in the lung were increased over the 8 wk period of experiment. The increased COHb and HO-1 were positively correlated with the increased plasma LPS and TNF-α level. Since increased HO-1 expression in intravascular macrophages and carbon monoxide overproduction are also reported in a model of common bile duct ligation in rats[52], it would be interesting to investigate the interplay of NOS/NO pathway and HO-1/CO pathway in pulmonary vascular abnormalities in cirrhotic rats. For this purpose, L-NAME (a non-selective inhibitor of NOS), AG (a selective inhibitor of iNOS), or ZnPP (a selective inhibitor of HO-1) were administrated in cirrhotic rats. Interestingly, we observed a mutual inhibition, i.e., L-NAME or AG inhibited HO-1 expression, whereas ZnPP inhibited eNOS and iNOS expression. Histologically, AG inhibitor likely inhibited the enlargement of diameters of the dilated pulmonary capillaries at the 6th wk but there was no obvious change in the number of capillaries (data not show). Therefore, CO may be another important mediator in the development of HPS, HO-1/CO and NOS/NO pathways may be interrelated in pathogenesis of intrapulmonary vascular abnormalities in cirrhotic rats.

The underlying mechanisms are required to be further investigated.

Hypoxia is very important in inducing expression of the vascular endothelial growth factor (VEGF)[54], which specifically regulates endothelial cell growth and differentiation and also acts as a survival factor for endothelial cells[55]. It is generally believed that hypoxia is a consequence of intrapulmonary vascular dilatation in the presence of IETM. Since hypoxia induces NO generation[56-58] and NO induces expression of VEGF[59-62], it is conceivable that blood vessel remodeling and angiogenesis or vasculogenesis occurs during the development of HPS in cirrhotic rats. However, it is worth mentioning that CO may also be a contributing factor in regulating the expression of VEGF since CO levels are correlated with the number of capillaries.

In summary, IETM plays a central role in the development of HPS under conditions of hepatic cirrhosis[63,64]. Therefore, strategies against LPS should be made for preventing the development of HPS.

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COMMENTS

Background

Hepatopulmonary syndrome (HPS) develops when arterial oxygenation deficiency occurs due to intrapulmonary vascular dilatation often associated with severe hepatic disease. Recent studies support that the presence of HPS significantly increases mortality in cirrhosis patients, particularly in those with decompensated liver disease. The pathogenesis of HPS remains to be elucidated. Some complications of cirrhosis, including hepatorenal syndrome, hepatic encephalopathy and HPS are closely associated with intestinal endotoxemia (IETM) in both experimental and clinical investigations. We further explored the pathogenesis of HPS on the basis of the preceding research in a cirrhotic rat model induced by multiple pathogenic factors.

Research frontiers

One of the characteristics of hepatopulmonary syndrome is intrapulmonary vascular dilation which severely affects pulmonary gas exchange leading to hypoxia and increased mortality of cirrhotic patients. The present study focused on clarifying the pathogenesis of HPS, particularly the mechanism of intrapulmonary vasodilation. Clinically, many studies have been performed on evaluating the prevalence, etiology, clinical features, early diagnosis, treatment and prognosis of this syndrome worldwide.

Innovations and breakthroughs

In this study, we further characterized the relation between severity of HPS and degree of hepatic dysfunction, and explored how IETM affects the development of HPS in cirrhotic rats. Our results indicate that the severity of HPS is directly associated with the level of blood LPS and progression of hepatic dysfunction, and IETM plays a central role in the development of HPS.

Applications

All of our findings in the present study further confirm that IETM plays a central role in the development of HPS under conditions of hepatic cirrhosis. Therefore, the strategy against LPS is certainly effective both in preventing the development of HPS and in its treatment.

Terminology

Angiogenesis: a natural process in the body that involves the growth of new blood vessels. It can occur in many diseases, such as coronary artery disease, peripheral artery disease and stroke when blood supply and oxygen are insufficient in tissues. It denotes the formation of new blood vessels from pre-existing ones. Vasculogenesis: the process of blood vessel formation occurring by a *de novo* production of endothelial cells. Microvasculature remodeling: alterations in a blood vessel network resulting from arteriogenesis and angiogenesis. Briefly, arteriogenesis is an increased arterial diameter while angiogenesis is an increased number of capillaries either by sprouting from or splitting existing capillaries. External events stimulate these two types of vessel growth through a combination of mechanical and chemical pathways.

Peer review

This manuscript is very interesting. The topic is original for a basic science study and the structure of material and methods is outstanding. Results are clearly explored and discussion widely covers the topic even for humans.

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CLINICAL RESEARCH

Two distinct pathways of p16 gene inactivation in gallbladder cancer

Hiroyuki Tadokoro, Takako Shigihara, Tomomi Ikeda, Masaru Takase, Masafumi Suyama

Hiroyuki Tadokoro, Masafumi Suyama, Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 136-0075, Japan
Takako Shigihara, Tomomi Ikeda, Biochemical Research Center, Division of Molecular and Biochemical Research, Juntendo University School of Medicine, Tokyo 136-0075, Japan
Masaru Takase, Department of Human Pathology, Juntendo University School of Medicine, Tokyo 136-0075, Japan
Correspondence to: Hiroyuki Tadokoro, MD, Department of Gastroenterology, Juntendo Tokyo Koto Geriatric Medical Center, 3-3-20 Shinsuna, Koto-ku, Tokyo 136-0075, Japan. heroyou27@yahoo.co.jp
Telephone: +81-356-323111 Fax: +81-356-323728
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Abstract

AIM: To examine the mechanism of inactivation of the p16 gene in gallbladder cancer, and to investigate p16 alterations and their correlation with clinicopathological features.

METHODS: Specimens were collected surgically from 51 patients with gallbladder cancer. We evaluated the status of protein expression, loss of heterozygosity (LOH), homozygous deletion and promoter hypermethylation using immunohistochemistry, microsatellite analysis, quantitative real-time polymerase chain reaction (PCR) and methylation-specific PCR, respectively. In addition, mutations were examined by direct DNA sequencing.

RESULTS: Homozygous deletions of the p16 gene exon2, LOH at 9p21-22, p16 promoter hypermethylation, and loss of p16 protein expression were detected in 26.0% (13/50), 56.9% (29/51), 72.5% (37/51) and 62.7% (32/51), respectively. No mutations were found. LOH at 9p21 correlated with the loss of p16 protein expression ($P < 0.05$). Homozygous deletion of the p16 gene, a combination LOH and promoter hypermethylation, and multiple LOH at 9p21 were significantly correlated with the loss of p16 protein expression ($P < 0.05$). LOH at 9p21 and promoter hypermethylation of the p16 gene were detected in 15.4% (2/13) and 92.3% (12/13) of the tumors with homozygous deletion of the p16 gene, respectively. P16 alterations were not associated with clinicopathological features.

CONCLUSION: Our results suggest that LOH and homozygous deletion may be two distinct pathways in the inactivation of the p16 gene. Homozygous deletion, a combination of LOH and promoter hypermethylation, and multiple LOH are major mechanisms of p16 inactivation in gallbladder cancer.

Key words: Gallbladder cancer; Homozygous deletion; Loss of heterozygosity; p16; Quantitative real time PCR

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INTRODUCTION

Gallbladder carcinoma is a highly malignant neoplasm with a poor prognosis, and most patients are diagnosed at an already advanced stage[1-3]. Gallbladder carcinoma is a relatively common cancer and is the sixth highest cause of cancer death in Japanese women.

Several genes have been implicated in the tumorigenesis of gallbladder cancers, including *K-ras*, cerbβ2, p53[4,5], $p16^{INK4a}$/CDKN2, and the fragile histidine triad (FHIT)[6]. Genetic alterations in the 9p21 chromosomal region have been linked to malignant progression.

The p16 gene, located on chromosome 9p21, encodes a critical negative regulator of cell cycle progression and is inactivated in various cancers. The p16 gene is an important tumor suppressor gene, which interacts strongly with cyclin-dependent kinases 4 and 6, and inhibits their ability to interact with cyclin D[7]. p16 induces cell cycle arrest at G1 and G2/M checkpoints, which blocks cells from phosphorylating retinoblastoma protein 1, and prevents cells from exiting the G1 phase of the cell cycle[8]. p16 can act as a negative regulator of normal cell proliferation. Inactivation of the p16 gene plays an important role in tumorigenesis. p16 inactivation by loss of heterozygosity (LOH) and point mutations has been reported in biliary tract cancers[9] and intrahepatic cholangiocarcinoma[10].

Aberrant promoter methylation is an important mechanism in silencing cancer-related genes during the process of carcinogenesis. Epigenetic inactivation of tumor suppressor genes has been commonly reported in various tumors[11]. Promoter hypermethylation, as

well as gene deletions and point mutations, has been shown to be a major mechanism of p16 inactivation[12,13]. Hypermethylation of the CpG islands of the p16 gene promoter region has been reported in various types of tumor.

The main modes of p16 gene inactivation in gallbladder carcinoma are known to include LOH, mutation and hypermethylation[12,14]. Homozygous deletion of the p16 gene has not previously been investigated in gallbladder cancer. Therefore, we sought to comprehensively study genetic and epigenetic alterations of p16, including homozygous deletion of the p16 gene, and the relationship between these abnormalities and clinicopathological features.

MATERIALS AND METHODS

Tissue specimens

Paraffin-embedded tissue samples were obtained from 51 patients who underwent surgical resection at Juntendo University School of Medicine, Japan, between April 1996 and April 2005. Gallbladder carcinoma patients consisted of 25 women and 26 men, ranging in age from 36 to 94 (mean, 65.1) years. Their tumors consisted of 46 adenocarcinomas and five adenosquamous carcinomas. The adenocarcinomas included 39 well-to-moderately differentiated and seven poorly differentiated tumors. Most of the patients had advanced gallbladder carcinoma, with invasion of the subserosa (n = 22, 43.1%) and serosa (n = 18, 35.3%), while the other 11 patients (19.6%) had early gallbladder carcinoma (mucosa or muscularis propria invasion). All histological slides were reviewed by M.T. and H.T. and were classified based on the WHO classification of gallbladder carcinoma. Medical records were available for all patients.

Immunohistochemical analysis

Immunohistochemistry was performed using anti-p16 (F-12; 1:500 dilution; Santa Cruz Biochemistry, Santa Cruz, CA, USA) and an automated slide staining system (NexES IHC; Ventana, AZ, USA), according to the manufacturer's instructions. P16 immunostaining was performed within 2 weeks of sectioning, because reactivity decreased over time after preparation. Normal lymphocytes and intrahepatic bile ducts were positive controls for p16. The percentage of positive nuclei was scored as follows: -, 0%-10%, + 1%-25%, ++, 25%-50%, and +++, > 50% positive cells. Scores of +, ++ and +++ were considered to represent positive immunostaining, while - was deemed to be negative.

DNA extraction

Formalin-fixed, paraffin-embedded tissue blocks were used. Serial 10-μm sections were cut from each block and stained with hematoxylin and eosin to locate the tumor and non-neoplastic tissue before DNA extraction. Sections were cut, deparaffinized, and microdissected with an 18-gauge needle. The microdissected tissues were digested overnight at 55℃ in buffer (1% Tween 20, 10 mmol/L Tris-HCl, pH 8, 1 mmol/L EDTA, and 100 μg/mL proteinase K). The lysates were heated at 95℃ for 10 min and stored at 4℃ until analyzed by polymerase chain reaction (PCR).

Methylation assay

DNA methylation was investigated using an EZ DNA methylation kit (Zymo Research, CA, USA), according to the manufacturer's protocol. Microdissected genomic DNA (1 ng) was denatured with M-dilution buffer at 37℃ for 15 min, followed by incubation with CT conversion reagent at 50℃ for 16 h in the dark. After treatment, the DNA was purified using M-binding buffer, incubated with M-desulphonation buffer at room temperature for 15 min, washed with wash buffer, and finally resuspended in M-elution buffer.

Primers for the p16 gene were 5'-TTATTAGAGGG TGGGGTGGATTGT-3' (sense) and 5'-CCACCTAAAT CAACCTCCAACCA-3' (antisense) for the unmethylated reactions, and 5'-TTATTAGAGGGTGGGGCGGATCG C-3' (sense) and 5'-GACCCCGAACCGCGACCGTAA-3' (antisense) for the methylated reactions, as described previously[15]. PCR reactions were started by denaturation at 95℃ for 5 min, followed by 40-45 cycles of 94℃ for 30 s, 65℃ (for methylated p16) or 60℃ (for unmethylated p16) for 45 s, and 72℃ for 60 s, with a final extension at 72℃ for 10 min. PCR products were analyzed by electrophoresis on a 3% agarose gel, and visualized by ethidium bromide staining. DNA from the Raji cell line was used as a positive control. Distilled water was used as a negative control.

LOH analysis

Paired normal and tumor DNA samples were amplified by hot-start PCR, using locus-specific flanking primer pairs for five fluorescently labeled microsatellite markers, D9S171-FAM, D9S1748-FAM, D9S942-NEX, D9S974-NED, and D9S1749-NED (Figure 1). Primer sequences were obtained from the NCBI UniSTS database (http://www.ncbi.nlm.nih.gov/). Markers mapping to the chromosome 9p21-22 region were used. D9S1748, D9S942 and D9S974 are within a coding sequence of the p16 gene. D9S1749 is telomeric to p16 and D9S171 is centromeric to p16. PCR was performed with an initial denaturation at 95℃ for 15 min, followed by 36 cycles of denaturation at 95℃ for 30 s, annealing at 58℃ (D9S171, D9S1748, D9S942, D9S974) or 55℃ (D9S1749) for 45 s, and extension at 72℃ for 60 s, and final extension at 72℃ for 10 min. After PCR, samples were diluted at 1:7 in formamide, heated to 95℃ for 2 min, chilled on ice, and analyzed with Genescan software on an ABI PRISM 310 genetic analyzer (PE-Applied Biosystems, Foster City, CA, USA). Allelic ratios in both normal and tumor samples were calculated and compared. The area under each peak, representing each allele in the microsatellite pair, was obtained. LOH was defined as a > 50% reduction in the tumor peak compared to that of the corresponding normal tissue. Additional bands that were not seen on normal DNA, but were observed in tumor samples, were considered evidence of microsatellite instability (MSI).

Detection of homozygous deletion of p16 exon2

Homozygous gene deletion and gene dosage of p16 exon2

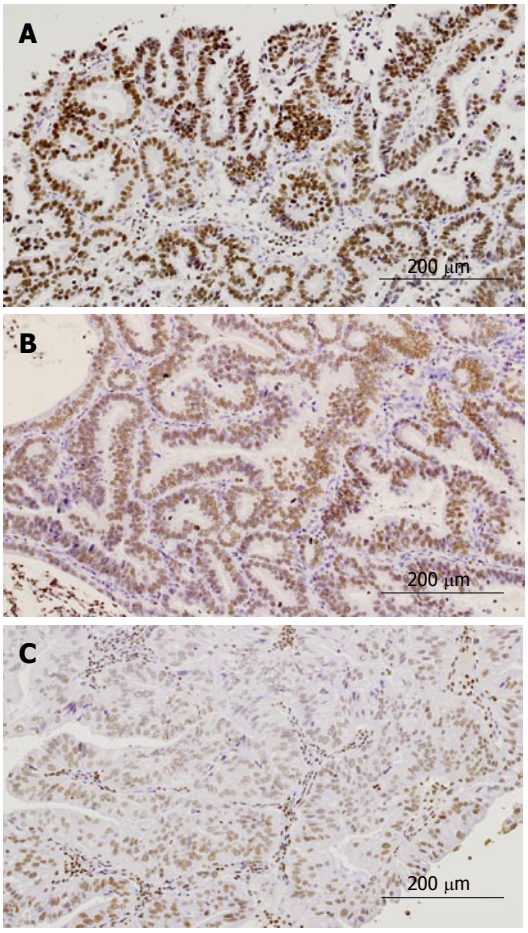

Figure 1 Immunohistochemical staining of p16. **A**: Tumor cells showing diffuse strong positive staining of p16; **B**: Tumor cells showing diffuse moderate staining; **C**: Tumor cells showing partly weak staining.

in gallbladder cancer were determined using a TaqMan-based real-time PCR method. Briefly, p16 gene exon2 and the GAPDH gene were amplified in a monoplex assay. The primer sequences for detecting p16 exon2 were 5'-AGCTTCCTTTCCGTCATGC-3' (sensze) and 5'-TCATGACCTGCCAGAGAGAA-3' (antisense). The primer sequences for detecting the GAPDH gene were 5'-GCATCCTGGGCTACACTGAG-3' (sense) and 5'-AG GTGGAGGAGTGGGTGTC-3' (antisense). The probe sequence for p16 gene exon2 was FAM-TGGCTCTG, and the probe sequence for the GAPDH gene was FAM-CTCCTCTG (the three FAM-labeled probes were manufactured by Roche Applied Science, Mannheim, Germany). The real-time PCR was performed in a 25 μL final volume containing 12.5 μL of Premix Ex Taq (Perfect Real Time; Takara, Kyoto, Japan), 50 ng DNA template, 10 μmol/L of each primer, 10 μmol/L Universal Library Probe, 0.5 μL ROX reference dye (50 ×), and 5.75 μL distilled water. The thermal cycling conditions on the ABI PRISM 7500 instrument were set to 10 s at 95℃, followed by 40 cycles of 5 s at 95℃, alternating with 34 s at 60℃. DNA from lymphocytes isolated from a paraffin block was used as a positive control. All reactions were done in triplicate. Normalized gene dosage ratios were interpreted as follows: 0-0.3, homozygous deletion; 0.31-0.69, under-representation (of the test gene, relative to the reference gene); 0.7-1.49, retention of copy number; and > 1.5 over-representation.

Table 1 The primer sequence of the p16 genes

Exon	Primer sequence	Size (bp)	Anneling temperature (℃)
Exon1α	F: 5'-GAGAGGGGGAGAGCAGGCAG-3' R: 5'-GCAAACTTCGTCCTCCAGAGT-3'	250	58
Exon2-1	F: 5'-AGCTTCCTTTCCGTCATGC-3' R: 5'-GCAGCACCACCAGCGTG-3'	203	56
Exon2-2	F: 5'-AGCCCAACTGCGCCGAC-3' R: 5'-CCAGGTCCACGGGCAGA-3'	147	58
Exon2-3	F: 5'-TGGACGTGCGCGATGC-3' R: 5'-GGAAGCTCTCAGGGTACAAATT C-3'	189	56
Exon3	F: 5'-CCGGTAGGGACGGCAAGAGA-3' R: 5'-CTGTAGGACCCTCGGTGACTGAT GA-3'	169	58

Mutation analysis

Mutation analysis was performed for the p16 gene (exons 1α, 2 and 3). Primer sequences and PCR conditions were as described previously[16,17] (Table 1). Amplification was performed using a Perkin Elmer GeneAmp 9600 Thermal cycler. After visualizing the PCR products in a 3% agarose gel, an aliquot (5 μL) of the PCR product was treated at 37℃ for 15 min with 1 μL ExoSAP-IT (GE Healthcare Biosciences, Piscataway, NJ, USA), followed by inactivation at 80℃ for 15 min. Part of this mix (6 μL) was directly sequenced using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems Japan, Chiba) on an automated sequencer (ABI PRISM 3100 Genetic analyzer; Perkin-Elmer, Japan).

Statistical analysis

Frequency distributions were analyzed by the χ^2 test. Correlations were examined between alterations of p16 and p16 expression, or between alterations of p16 and clinicopathological parameters. $P < 0.05$ was deemed statistically significant.

RESULTS

Immunohistochemical analysis

Loss of p16 protein expression occurred in 62.7% (32/51) of gallbladder cancer patients. Staining for p16 was weak in 13.7% (7/51) of patients, moderate in 17.6% (9/51), and strong in only 9.8% (5/51) of patients (Figure 1). There was no significant difference in p16 protein expression according to patient age, gender, tumor stage, T factor, N factor or histology (Table 2).

p16 status

LOH at 9p21-22 was detected in at least one marker in 56.9% (29/51) of patients. LOH occurred in 11.8%, 27.5%, 11.8%, 13.7% and 39.2% of patients who were positive for the markers D9S171, D9S1748, D9S942, D9S974 and D9S1749, respectively. Approximately 29.4% of the cases presented with LOH in a single marker, 11.8% with LOH in two markers, and 15.7% with LOH in three or more markers. LOH at the three markers D9S1748,

Table 2 Association between p16 alterations and clinicopathological variables in gallbladder cancers

Clinicopathological variables	p16 protein expression			Methylation of $p16^{INK4a}$			LOH at 9p21-22			Homozygous deletion of p16 gene		
	Absent	Present	*P*	Absent	Present	*P*	Retension	LOH	*P*	Absent	Present	*P*
	n	*n*		*n*	*n*		*n*	*n*		*n*	*n*	
Age												
< 65	13	7	0.789	5	15	0.753	10	10	0.427	13	6	0.481
> 65	19	12		9	22		12	19		24	7	
Gender												
Female	14	11	0.329	6	19	0.588	10	15	0.657	20	4	0.148
Male	18	8		8	18		12	14		17	9	
Tumor type												
Adenocarcinoma	28	18	0.401	14	32	0.148	19	27	0.423	35	10	0.068
Adenosquamous	4	1		0	5		3	2		2	3	
Differentiation grade												
Well-Moderate	22	17	0.144	12	27	0.907	18	21	0.115	29	10	0.16
Poor	6	1		2	5		1	6		6	0	
Stage												
0 and ⅠA, ⅠB	15	13	0.135	9	19	0.407	15	13	0.097	20	7	0.99
ⅡA and ⅡB	17	6		5	18		7	16		17	6	
T factor												
Tis and T1	6	6	0.296	3	9	0.828	8	4	0.060	11	1	0.11
T2 and T3	26	13		11	28		14	25		26	12	
N factor												
N0	21	16	0.150	9	28	0.416	19	18	0.054	28	8	0.329
N1	11	3		5	9		3	11		9	5	

All *P* values were revealed by χ^2-test.

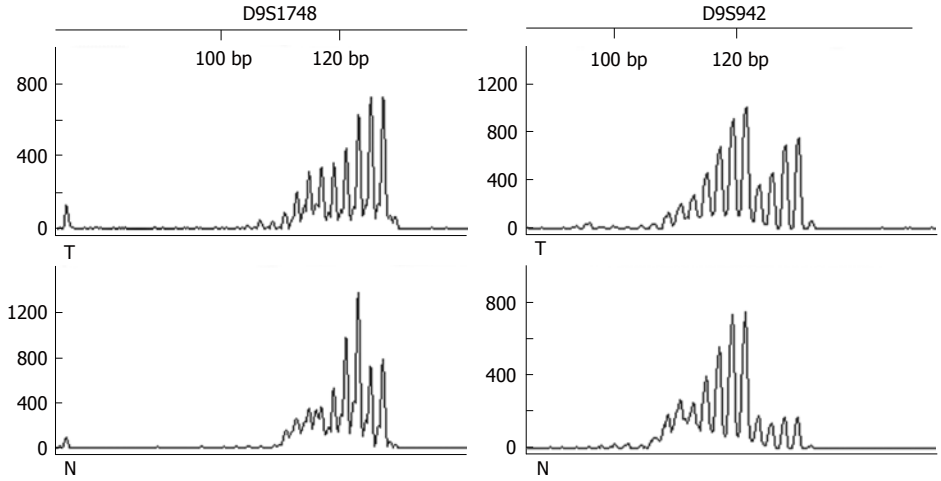

Figure 2 Representative example of the results of microsatellite analysis showing LOH at D9S1748 and LOH at D9S942 (right, case 25; left, case 45). The scales on the top and left side of each figure represent the size (bp) and the intensity, respectively. N: Normal; T: Tumor.

D9S942 and D9S974 within a coding sequence of the p16 gene was 37.3% in total. Representative examples of LOH at D9S942 and D91748 are shown in Figure 2.

Gene dosage of p16 exon 2 was successfully measured in 50 of 51 cases. Homozygous deletion of p16 exon2 was detected in 13 of 50 (26%) tumors. Overall the p16 gene was altered by homozygous deletion and LOH in 56.9% (29/51) of the tumors, indicating that alterations at this locus are involved in the vast majority of the tumors. In our analysis, LOH at 9p21-22 and homozygous deletion of p16 exon 2 were not associated with demographic variables such as age, gender, tumor histology and stage, T factor or N factor (Table 2).

p16 methylation status

Hypermethylation of the p16 gene was observed in 72.5% (37/51) of the patients (Figure 3). The relationship between p16 hypermethylation and various clinicopathological features was analyzed statistically. There was no significant correlation with the clinicopathological parameters assessed, including age, gender, tumor stage, T factor or N factor (Table 2). P16 methylation was found in 37 of 51 gallbladder cancer patients (72.5%), and loss of p16 protein expression was detected in 20 of the 37 tumors (54.1%) showing p16 hypermethylation. Our data showed that P16 protein expression was not significantly correlated with p16 hypermethylation.

p16 mutations

In tumors without homozygous deletions, exons 1α, 2 and 3 were amplified. In five cases, which were non-informative for the markers investigated, constitutive DNA was not available. No mutations were detected in exons 1α, 2 or 3.

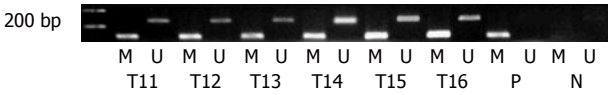

Figure 3 Methylation status of the $p16^{INK4a}$ gene by methylation-specific PCR. PCR products amplified by unmethylated (U) and methylated (M) specific primers. P: Positive control; N: Negative control. Methyaltion band is at 150 bp and unmethylated band is at 234 bp.

Table 3 Association between p16 protein expression and 9p21-22

	p16 protein expression		
	Negative	Positive	*P*
LOH (-)	12	10	
LOH (+)	20	9	0.292

OH was estiomated by allelic status at D9S171, D9S1748, D9S942, D9S974, D9S1749. LOH: Loss of heterozygosity.

Association between p16 protein expression and alterations of p16

We investigated the association between p16 protein expression and P16 alterations. LOH at 9p21-22 was not associated with the loss of p16 expression. However, LOH at three genes (D9S1748, D9S942 and D9S974) within a coding sequence of p16 was correlated with the loss of p16 expression ($P < 0.05$) (Tables 3 and 4). Furthermore, homozygous deletion of the p16 gene, a combination of LOH at 9p21 and promoter hypermethylation of the p16 gene, multiple LOH at 9p21 correlated with the loss of p16 protein expression ($P < 0.05$) (Table 5). Loss of p16 protein expression was detected in nine of 13 tumors with homozygous deletion. LOH at 9p21 was detected in only two of 13 cases with homozygous deletion, while promoter hypermethylation of the p16 gene was detected in 12 of 13 cases with homozygous deletion.

DISCUSSION

We examined the mechanisms of p16 inactivation and the relationship between p16 alterations and clinicopathological features in gallbladder cancer. p16 alterations have been evaluated individually in previous studies, but our study is believed to be the first attempt to evaluate homozygous deletion of the p16 gene. We sought to carry out a comprehensive study of p16 gene status, investigating gene dosage, allelic status, hypermethylation, mutation, protein expression, and clinicopathological features in gallbladder cancer.

Loss of p16 protein expression has been examined in various types of cancer, including gallbladder cancer. We examined p16 protein expression by immunohistochemistry in 51 cases of gallbladder cancer. Loss of p16 protein expression has been reported to range between 24 and 76% in gallbladder cancer[14,18-20,23]. In our study, the loss of p16 expression was detected in 32 of 51 (62.7%) patients with gallbladder cancer. In addition, the loss of p16 expression has been correlated with tumor progression or with decreased survival among patients with carcinoma of the lung, pancreas and esophagus, and malignant melanoma[21,22,24]. The correlation between p16 expression and clinicopathological factors is controversial. Ma *et al*[15] have reported that decreased expression of p16 is correlated with pathological grade and tumor progression in gallbladder carcinoma. However, Shi *et al*[16] have reported that loss of p16 protein expression is not significantly associated with any clinicopathological factors or survival. Quan *et al*[20] have reported that the loss of p16 expression is not associated with pathological grade. We also failed to find any association between the loss of p16 expression and clinicopathological parameters.

The mechanisms of inactivation of the p16 gene are homozygous deletion, LOH, promoter hypermethylation, rearrangement, and intragenic mutation.

Homozygous deletions are important for complete inactivation of tumor suppressor genes[13]. Previous investigators have evaluated homozygous deletion of the p16 gene in a small series of biliary tract and gallbladder cancer cell lines. Ku *et al*[25] have reported that homozygous deletion of the p16 gene was detected in three of six (50%) gallbladder cell lines. Yoshida *et al*[9] have reported that homozygous deletion of the p16 gene was detected in one of two gallbladder cell lines and in two biliary tract cell lines. Caca *et al*[26] have reported that homozygous deletion of the p16 gene was detected in eight of nine (88.8%) biliary tract cell lines, but homozygous deletion of the p16 gene was not detected in 21 biliary tract cancers. Homozygous deletion of the p16 gene has not previously been examined in gallbladder cancer. Our study is believed to be the first report to evaluate homozygous deletion of the p16 gene. We employed quantitative real-time PCR to evaluate homozygous deletion. In our series, homozygous deletion of the p16 gene was detected in 13 of 50

Table 4 Association between p16 protein expression and LOH at 9p21

	p16 protein expression		
	Negative	Positive	*P*
LOH (-)	16	16	
LOH (+)	16	3	0.0146

LOH was estiomated by allelic status at D9S1748, D9S942, D9S974.

Table 5 Association between p16 immunohistochemistry and p16 alterations in gallbladder cancers

	p16 immunohistochemistry		
	Positive	Negative	*P*
Retension	3	3	
Hypermethyaltion	9	6	
LOH	1	3	
LOH (multiple) + Hypermethylation	2	8	
Multiple LOH	0	3	
Homozygous deletion	4	9	$P < 0.05$

LOH: Loss of heterozygosity; Multiple LOH: LOH in 9p21 at more than two loci.

cases (26%). Previous studies have demonstrated that homozygous deletion of tumor suppressor genes plays an important role in the development and progression of some malignancies. However, in our series, homozygous deletion of the p16 gene was not associated with clinicopathological features.

Loss of p16 expression is correlated with homozygous deletion of the p16 gene in gallbladder cancer and other malignancies. Eight of nine biliary tract cell lines with homozygous deletion of the p16 gene showed loss of p16 expression, as reported by Caca *et al*[26]. In our series, loss of p16 expression correlated with homozygous deletion of the p16 gene in nine of 13 tumors. In four cases, homozygous deletion of the p16 gene did not correlate with p16 protein expression. Four tumors with homozygous deletion of the p16 gene displayed moderate to strong positive staining in immunohistochemistry. These tumors showed diffuse positive staining in some areas and partial or complete loss of p16 staining in other areas. The areas which showed loss of p16 expression were not captured during microdissection, and consequently the tumors were scored as having homozygous deletion. Previous reports have shown that the loss of p16 protein expression does not always correlate with homozygous deletion of the p16 gene[27,28].

Promoter hypermethylation of p16 leads to inactivation of the gene in various cancers. In gallbladder cancer, the frequency of p16 promoter hypermethylation ranges from 24% to 56%[12,29-31]. In our study, p16 hypermethylation was found in 72.5% (37/51) of the tumors. The frequency in our study was comparatively higher than that in previous studies. Previous studies have revealed that the frequency of p16 promoter hypermethylation is not associated with tumor progression and clinicopathological characteristics[30,31]. Similarly, we found that p16 hypermethylation was not associated with any clinicopathological features. Some investigators have demonstrated that p16 hypermethylation is correlated with the loss of p16 expression in intraductal carcinoma of the liver, lung cancer, hepatocellular carcinoma and esophageal cancer[32-35]. In the present study, 20 of 30 (66.6%) cases with p16 promoter hypermethylation showed a loss of p16 expression. However, there was no correlation between promoter hypermethylation and the loss of p16 expression.

We failed to detect any p16 mutations in the present study. Previous studies have shown a frequency of p16 mutation of 0%-80% in gallbladder and biliary tract cancer and cell lines[9,12,14,25]. Ueki *et al* have reported that 13 of 53 (24.5%) cases of gallbladder cancer showed non-silent p16 gene mutations. Kim *et al* have reported that p16 mutations were detected in four of 13 (30.7%) patients with gallbladder cancer. Yoshida *et al* have reported that eight of 10 cases of gallbladder cancer showed p16 point mutations. These studies did not examine homozygous deletion of the p16 gene. Ku *et al* have reported that homozygous deletion of the p16 gene was found in three of six biliary tract cell lines, but no p16 mutation was found in the remaining three biliary tract cell lines, which did not show homozygous deletion. Caca *et al*[26] have reported that p16 mutations were not found in three biliary tract cell lines and 21 biliary tract cancers, which did not show homozygous deletion of the p16 gene. In the present study, a p16 mutation was not found in any of the cases analyzed. These results suggest that the p16 mutation is associated with homozygous deletion of the p16 gene.

LOH at 9p21 has been detected in different types of tumors and cell lines[36,37]. The frequency of LOH at 9p21 in gallbladder carcinoma ranges from 38 to 60%[5,38,39]. Previous studies have demonstrated that LOH at 9p21 correlates with the loss of p16 expression in various types of cancer[22,40]. We also investigated the association between p16 protein expression and LOH at 9p21-22 in gallbladder cancer. Although an association between LOH at 9p21-22 and p16 protein expression was not found in our study, LOH at three genes (D9S1748, D9S942 and D9S974) which are located within a coding sequence of p16, correlated with loss of p16 protein expression. The mode of p16 silencing may be explained by a modification of Knudson's two-hit model[41]. In cases which show the loss of p16 protein expression, LOH or promoter hypermethylation may have occurred in only one allele, and other mechanisms may also have been involved in other alleles.

In conclusion, we investigated comprehensively the mechanisms of inactivation of the p16 gene in gallbladder cancer, and the association between p16 alterations and clinicopathologic features. Although the mutation of p16 is a rare event in gallbladder cancer, homozygous deletion, LOH and promoter hypermethylation were frequent events. LOH at 9p21 correlated with loss of p16 protein expression. In addition, homozygous deletion of the p16 gene, combination of LOH and promoter hypermethylation, and multiple LOH at 9p21 significantly correlated with loss of p16 protein expression ($P < 0.05$). LOH at 9p21 was detected in only two of 13 cases with homozygous deletion, while promoter hypermethylation of the p16 gene was detected in 12 of 13 cases with homozygous deletion. Promoter hypermethylation of the p16 gene may have occurred as an earlier event, followed by homozygous deletion as a later event in cases of homozygous deletion. LOH and homozygous deletion may be two distinct pathways for inactivating the p16 gene in gallbladder cancer.

Our results suggest that multiple alterations of the p16 gene imply multiple mechanisms for the inactivation of the p16 gene in gallbladder cancer. The mechanisms may be important for the diagnosis and treatment of this disease.

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CLINICAL RESEARCH

Left-sided gallbladder: Its clinical significance and imaging presentations

Sheng-Lung Hsu, Tai-Yi Chen, Tung-Liang Huang, Cheuk-Kwan Sun, Allan M Concejero, Leo Leung-Chit Tsang, Yu-Fan Cheng

Sheng-Lung Hsu, Tai-Yi Chen, Tung-Liang Huang, Leo Leung-Chit Tsang, Yu-Fan Cheng, Department of Diagnostic Radiology, Chang Gung Memorial Hospital - Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan, China
Cheuk-Kwan Sun, Allan M Concejero, Department of Surgery, Chang Gung Memorial Hospital - Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan, China
Supported by Project Grant NHRI-GT-EX92-9228SP from the National Health Research Institutes and NSC 93-2314-B-182A-084 from the National Science Council, Taiwan, China
Correspondence to: Yu-Fan Cheng, MD, Department of Diagnostic Radiology, Chang Gung Memorial Hospital - Kaohsiung Medical Center, Chang Gung University College of Medicine, Kaohsiung, Taiwan, 123 Ta-Pei Road, Niao-Sung, Kaohsiung, 833, Taiwan, China. cheng.yufan@msa.hinet.net
Telephone: +886-7-7317123-3027 Fax: +886-7-7331415
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Abstract

AIM: To assess the importance of preoperative diagnosis and presentation of left-sided gallbladder using ultrasound (US), CT and angiography.

METHODS: Retrospective review of 1482 patients who underwent enhanced CT scanning was performed. Left-sided gallbladder was diagnosed if a right-sided ligamentum teres was present. The image presentations on US, CT and angiography were also reviewed.

RESULTS: Left-sided gallbladder was diagnosed in nine patients. The associated abnormalities on CT imaging included portal vein anomalies, absence of umbilical portion of the portal vein in the left lobe of the liver, club-shaped portal vein in the right lobe of the liver, and difficulty in identifying segment Ⅳ. Angiography in six of nine patients demonstrated abnormal portal venous system (trifurcation type in four of six patients). The main hepatic arteries followed the portal veins in all six patients. The segment Ⅳ artery was identified in four of six patients using angiography, although segment Ⅳ was difficult to define on CT imaging. Hepatectomy was performed in three patients with concomitant liver tumor and the diagnosis of left-sided gallbladder was confirmed intraoperatively.

CONCLUSION: Left-sided gallbladder is an important clinical entity in hepatectomy due to its associated portal venous and biliary anomalies. It should be considered in US, CT and angiography images that demonstrate no definite segment Ⅳ, absence of umbilical portion of the portal vein in the left lobe, and club-shaped right anterior portal vein.

Key words: Left-sided gallbladder; Angiography; Computed tomography; Ultrasound

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INTRODUCTION

Left-sided gallbladder is defined as a gallbladder located to the left side of the ligamentum teres. This anomaly can be divided into three anatomic abnormalities: a situs inversus, an ectopic left-sided gallbladder, and a right-sided ligamentum teres[1-4]. Left-sided gallbladder with right-sided ligamentum teres has been a rare anomaly since Hochstetter's first description in 1886. Its characteristic description is a "gallbladder lying over the left side of the ligamentum teres"[1,4,5]. The associated anomalies with left-sided gallbladder include portal vein anomalies, biliary system anomalies, and segment Ⅳ atrophy[5-9]. The complex anomalies associated with left-sided gallbladder are important during hepatectomy, particularly in living donor liver transplantation. Preoperative survey of the anomalies of the portal triad is important in minimizing the incidence of postoperative biliary and vascular complications. Unknowingly, ligation of the left branch of the portal vein and bile duct that contribute to three-quarters of the liver hemodynamics will cause hepatic failure, biliary congestion and leakage[7,8,10-12]. This series reports nine cases of left-sided gallbladder with right-sided ligamentum teres. Its associated anomalies and significance during hepatectomy are identified and discussed.

MATERIALS AND METHODS

We reviewed 1528 enhanced CT scans performed

Table 1 Patient profiles and imaging results

Patient number	Clinical status and history	Club-shaped form portal vein (ultrasound finding[1])	Left lateral segment of liver (computed tomography finding[2])	Segment Ⅳ artery (angiogram finding)	Portal vein configuration (angio-portogram finding)
1	HCV, HCC, right hepatectomy	Over right lobe liver	Hypertrophic	From RHA	Trifurcation type
2	HCC, HBV and HCV	Not clear	Normal size	Not obvious	Other type
3	Right lobe HCC	Over right lobe liver	Mild hypertrophic	From RHA	Trifurcation type
4	Right lobe HCC, HBV	Over right lobe liver	Normal size	> From RHA	Trifurcation type
5	Hysterectomy, abdominal pain	Not clear	Normal size	NA	Not available
6	Liver tumor, HBV	Not clear	Mild hypertrophic	NA	Not available
7	Multiple HCC	Over right lobe liver	Normal size	NA	Not available
8	HCC, left lateral partial segmentectomy	Not clear	Normal size	From LHA	Trifurcation type
9	HCC, left lateral segmentectomy	NA	Normal size	Not obvious	Other type

[1]No umbilical portion over left lobe liver in all patients whose US results were available; [2]Club-shaped form portal vein over right side of the liver, no umbilical portion over left lobe of the liver. No defined segment Ⅳ in any patients. HBV: Hepatitis B carrier; HCV: Hepatitis C carrier; NA: Not available; RHA: Right hepatic artery.

for survey of liver tumors, other abdominal tumors, retroperitoneal tumors and abdominal pain at Chang Gung Memorial Hospital, Kaohsiung, Taiwan from 1999 to 2004. Forty-six patients were excluded because the gallbladder position or fossa could not be determined from previous hepatectomy or cholecystectomy. Of the remaining 1482 patients, nine were diagnosed to have left-sided gallbladder due to the presence of a right-sided ligament teres on enhanced CT. Among these patients, one underwent right hepatectomy and cholecystectomy for segment Ⅳ hepatocellular carcinoma (HCC). The other two patients underwent left lateral segmentectomy and partial hepatectomy for left lateral segment HCC, respectively.

RESULTS

Nine patients from a screening population of 1482 patients (0.6%) were diagnosed with left-sided gallbladder with enhanced CT. Ultrasound (US) was performed in eight of the nine patients, and angiography was done because of liver tumors in six patients. The clinical status and imaging findings are listed in Table 1. US demonstrated no umbilical portion of the portal vein over the left lobe of the liver in eight patients, of whom, four had an abnormal club-shaped portal vein over the right side of the liver (Figure 1A), whereas the other four patients did not clearly show a dilated portion of the portal vein on the right side of the liver. Enhanced CT demonstrated absence of the umbilical portion of the portal vein over the left lobe of the liver, and an abnormal umbilical portion of the portal vein over the right lobe of the liver, as in the US findings. Segment Ⅳ of the liver was difficult to define on CT imaging in all nine patients (Figure 1B). Angiography was performed in six of the nine patients. In four patients, the portal veins ramified to the right and left portal tributaries, and the left tributary of the portal vein diverged into lateral and medial portions. The medial portion veered to the ventral side and formed the umbilical portion, which finally joined the ligamentum teres (trifurcation type) (Figure 1C). The other two patients had the portal veins ramified to the right, and small left portal tributaries. In these patients, the right portal vein formed the umbilical portion with some tributaries, and finally joined the ligamentum teres. The type of portal vein ramification could not be defined from conventional descriptions in these two patients (Figure 2). The main hepatic arteries ran alongside with the main portal venous tributaries in all six patients, of whom four had well-defined segment Ⅳ artery (Figure 3). One patient underwent right hepatectomy and cholecystectomy due to HCC over the right lobe of the liver. Another two patients receive left lateral segmentectomy. In these cases, the left-sided gallbladder was well-demonstrated intraoperatively (Figure 1D).

DISCUSSION

Left-sided gallbladder with right-sided ligamentum teres is rarely reported. However, with advances in diagnostic imaging modalities, such as US, CT, MRI and angiography, left-sided gallbladder is increasingly being reported to be associated with right-sided ligamentum teres, accompanied by abnormal intrahepatic portal venous branching. An initial impression of left-sided gallbladder is made when the ligamentum teres is deviated to the right side, and a right-sided umbilical portion of the portal vein is present with difficulty in defining segment Ⅳ anatomy[5,6,8]. Our survey of nine cases is the largest report to date, and revealed an incidence of 0.6% of this anomaly, which is consistent with the previously reported incidence of 0.1%-1.2%[5,7,8,13,14].

There are several explanations for the development of left-sided gallbladder without situs inversus. First, the normal gallbladder bud may migrate to the left lobe instead of the right, and lies on the left side of the ligamentum teres[15]. In this situation, the portal vein, biliary tree and hepatic artery should be in the normal location and classified into ectopic gallbladder. Second, the gallbladder may develop directly from the left hepatic duct, and is accompanied by failure in development of the normal structure on the right side[7,15]. Our literature review

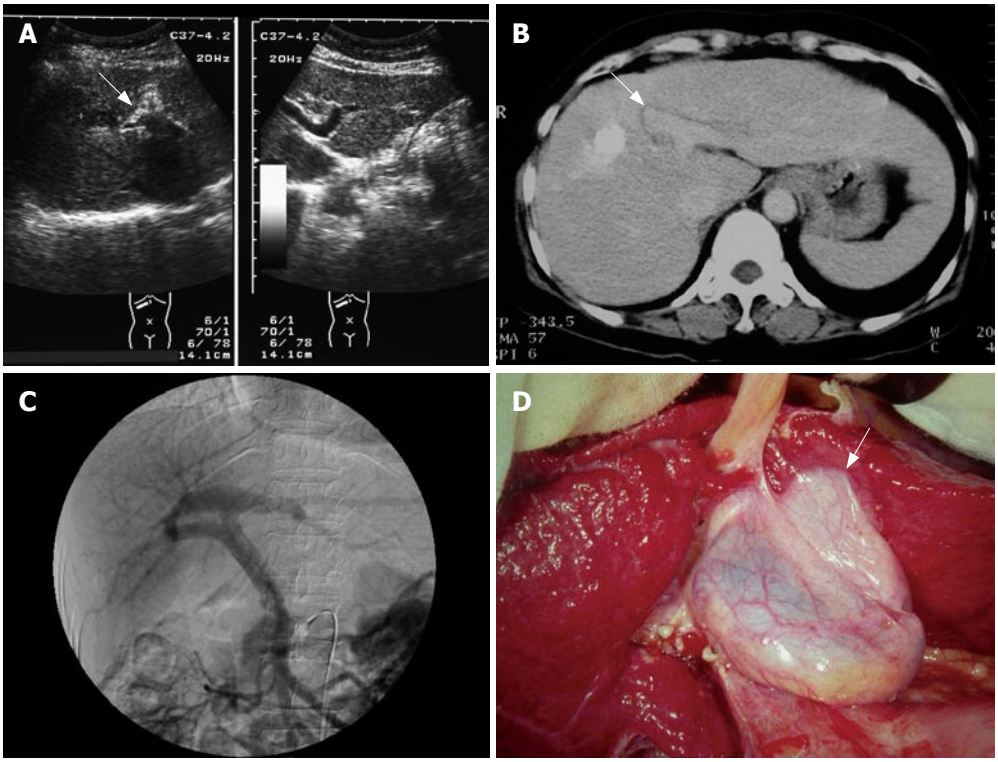

Figure 1 A 50-year-old woman with chronic hepatitis C. Right trisegmentectomy and cholecy-stectomy were performed due to the right lobe liver tumor. **A**: US demonstrated a club-shaped portal vein (arrow) over the right lobe of the liver and no umbilical portion over the left lobe of the liver; **B**: Enhanced CT demonstrated enlarged left lateral segment and a club-shaped portal vein (arrow) over the right side of the liver; **C**: Angiography demonstrated trifurcation type of the portal vein; **D**: Intraoperative photography demonstrated gallbladder over left side of ligamentum teres and adhesions on the left lateral segment (arrow).

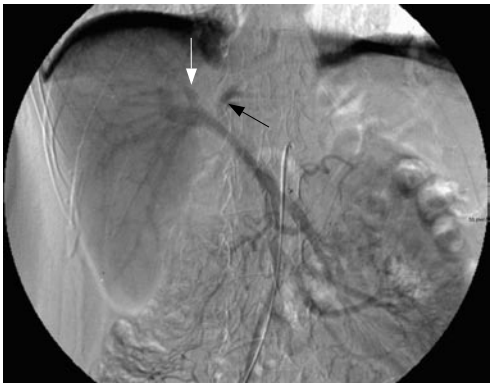

Figure 2 A 55-year-old man with HCC, chronic hepatitis B and C. Angio-portography demonstrated bifurcation with a small branch of the left portal vein (black arrow), segmental Ⅳ branch of the portal vein (white arrow), and a club-shaped form of the right portal vein with many branches.

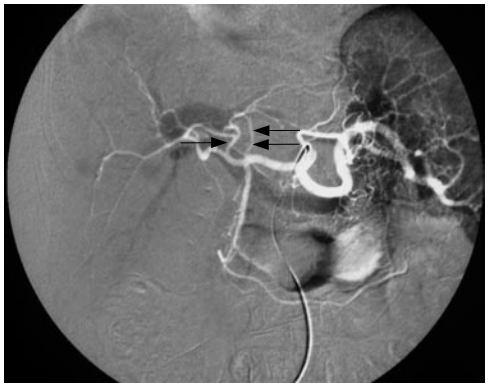

Figure 3 A 59-year-old woman with HCC in the segment Ⅵ of the liver and chronic hepatitis C. Angio-portography with substrate on arterial phase. The hepatic arteries (white vessels) followed the portal veins (black vessels). The segmental Ⅳ artery (arrow) was demonstrated. Right anterior side liver parenchyma was supplied from the segmental Ⅳ artery. The left hepatic artery (double arrows) originated from the common hepatic artery near the orifice of the gastroduodenal artery.

showed[5-7,11,15,16] only one case of left-sided gallbladder in which the cystic duct originated from the left hepatic duct[16]. The proposal of left-sided gallbladder development due to a second gallbladder originating directly from the left hepatic duct was not favored in those cases. Third, the left umbilical vein disappears and right umbilical vein partly remains. The peripheral (or placental) and central portions of the latter develop into the ligamentum teres and ligamentum venosum, respectively (Matsumoto's hypothesis)[13,17]. According to this hypothesis, the right umbilical portion should lie to the right of the gallbladder bed, but the gallbladder bed lay astride the ligamentum teres, as reported in four previous cases[7,18]. Nevertheless, this explanation also does not fit with our cases. Fourth, the gallbladder is on the left side of the ligamentum teres simply because the latter deviated to the right[5,7,16]. Maetini *et al*[7] have stated that there are three types of portal vein anomalies with the umbilical portion supplying the right anterior portion. In type A, the umbilical portion is to the right of the Cantlie line, whereas the umbilical portion lies on and to the left of the Cantlie line in types B and type C, respectively (Figure 4). They have also found that in the cases of left-sided gallbladder, the right posterior segmental duct joins the common duct distal to the bifurcation, and the aberrant right anterior segmental duct lies in the groove for the ligamentum teres, at which it forms a reverse U-shaped segment similar to the portal vein (Figure 5). They believe that the gallbladder on the left side of the ligamentum teres is there simply because the latter deviated to the right. As the right lobe of the liver becomes atrophied, we propose that the ligamentum teres moves from the left (type C) to the right (type A).

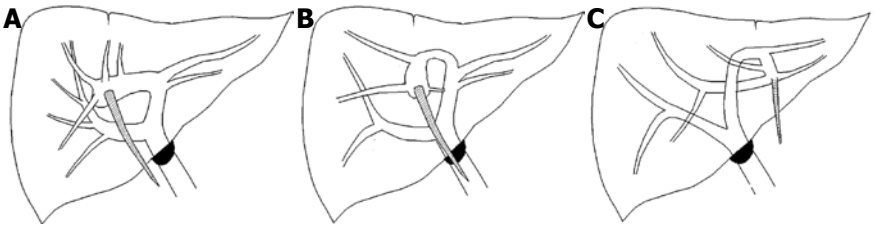

Figure 4 Maetini *et al* have described three types of portal vein anomalies with the umbilical portion supplying the right anterior portion. In type **A**, the umbilical portion is over the right side of the Cantlie line. In type **B**, the umbilical portion lies on the Cantlie line. In type **C**, the umbilical portion arises from the left portal vein with anterior segmental branches. As the ligamentum teres moves from the type C to the type A, the gallbladder moves to the left of the ligamentum teres.

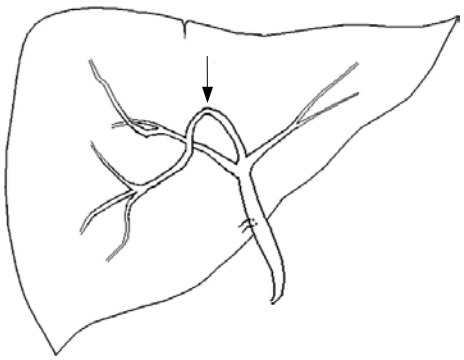

Figure 5 The right anterior segmental bile duct ran downward and ventrally formed a reverse U-shape (arrow), which followed the right anterior portal vein and lay in the groove for the ligamentum teres in left-sided gallbladder. Maetani *et al* believe that the gallbladder on the left side of the ligamentum teres is simply because the latter deviated to the right.

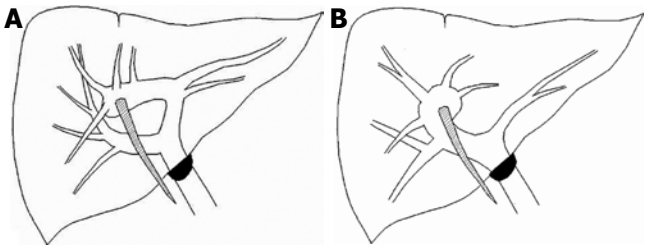

Figure 6 **A**: The trifurcation type of portal vein anomaly in a left-sided gallbladder; **B**: Bifurcation type of portal vein anomaly in a left-sided gallbladder. The abnormal intrahepatic portal venous branches could not be determined into these two types was divided into the other type.

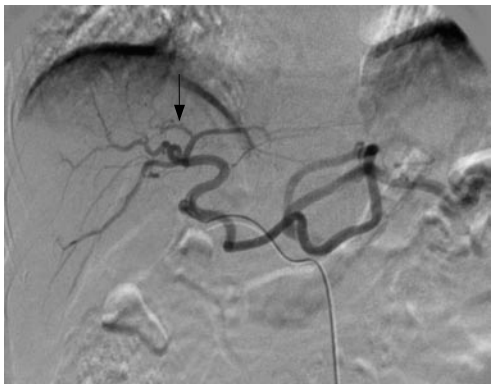

Figure 7 A 64-year-old man with HCC. The segmental Ⅳ artery (arrow) from the left hepatic artery is demonstrated.

The gallbladder moves to the left of the ligamentum teres, and the umbilical portion occupies the right side of liver.

Left-sided gallbladders have previously been reported to be associated with portal vein anomalies, biliary system anomalies, and segment Ⅳ atrophy. The associated portal vein anomalies are significant in hepatectomy, split liver transplantation, or living donor liver hepatectomy in transplantation, and can be classified into three types: the trifurcation type, bifurcation type and the other type, in which portal vein ramification cannot be defined from conventional descriptions (Figure 6)[5,19]. Intrahepatic arteries in left-sided gallbladders have not previously been described in detail[2,5]. In principle, the hepatic arteries follow the portal venous tributaries but the hepatic arterial variations and dissociation between the intrahepatic bile duct and portal vein have been reported[5,11,20,21]. In our six patients, in general, the hepatic arteries followed the portal venous tributaries (Figure 3). Angiography demonstrated the segment Ⅳ hepatic artery (Figures 3 and 7) in four of six patients, although segment Ⅳ was difficult to define on CT imaging. Segment Ⅳ atrophy is unlikely and we believe that the segment is embedded in the right lobe of the liver, and the true right lobe of the liver is relatively hypoplastic. Hypoplasia of the right lobe of the liver contributes to the ligamentum teres and umbilical portion being deviated to the right and therefore, causing a left-sided gallbladder. The dividing line between segment Ⅳ and the right lobe of the liver was not visualized, while segment Ⅳ was embedded in the right lobe of the liver. Although biliary tree anatomy was not evaluated in our patients, the bile duct and arterial branches appear to follow the portal venous tributaries of left-sided gallbladders[5,7,8,11]. If segment Ⅳ is embedded in the right lobe of the liver, the segment Ⅳ bile duct originated from left main intrahepatic duct ran downward and ventrally formed a reverse U-shaped right anterior segmental bile duct because of the intrahepatic duct following the portal vein. The segment Ⅳ bile duct can reasonably explain the formation of a reverse U-shaped right anterior segmental duct that joins the left hepatic duct and lies in the groove for the ligamentum teres, as reported by Maetani *et al* and Newcombe *et al*[7,22,23].

A left-sided gallbladder is an important entity during liver resection, particularly in left hepatectomy for tumors, complicated hepatolithiasis, and in liver transplantation[7,8,24]. Our experience of one patient who underwent hepatectomy for liver tumor in the segment Ⅳ with diagnosis of left-sided gallbladder and trifurcation type of portal vein anomaly aided in planning for resection (Figure 1A-D). As a result, an extended right instead of a left hepatectomy was performed. If this anomaly had not been recognized preoperatively, left hepatectomy could have been performed, and the portal vein supplying three-quarters of the liver may have been ligated, leading to

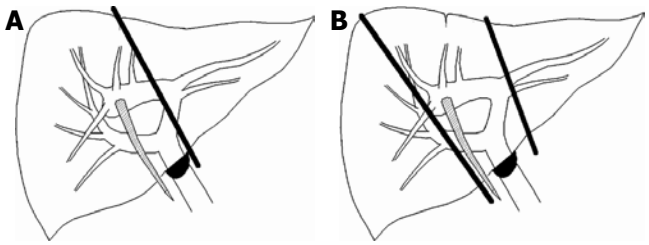

Figure 8 Trifurcation type of portal vein anomaly. **A**: If left hepatectomy is performed, the portal veins draining three-quarters of the liver parenchyma will be ligated. Hepatic necrosis may occur over right anterior portion of the liver parenchyma and contribute to hepatic failure; **B**: Left lateral segmentectomy or right hepatectomy is safe in this type portal vein anomaly.

hepatic failure, biliary congestion or bile leakage[7,8,11,12]. In two other patients, a tumor was located over the lateral segment; left hepatectomy was considered risky and only a left lateral segmental or partial hepatectomy was acceptable in both cases (Figure 8). All patients were discharged uneventfully post-hepatectomy.

Prior knowledge of a left-sided gallbladder is also important in living donor liver transplantation. In right lobe living donor liver transplantation, right lobe graft harvesting encounters double right portal veins in a patient with a left-sided gallbladder. Hepatic venoplasty or vein graft interposition for the reconstruction of the portal veins must be performed, thereby contributing to the complexity of surgery. In left lobe living donor liver transplantation, left-sided gallbladders with trifurcation-type portal vein anomaly are more technically demanding because only the left lateral segment can be used, and separation of the right anterior portal and left portal vein is mandatory[12,25-27]. The volume of the left lateral segment instead of the whole lateral lobe is calculated to determine left liver volume suitable for donation. In some cases, there is no common trunk of the left portal veins, which leads to difficulty in portal vein reconstruction and the possibility of portal vein thrombosis[8].

Anatomical variations associated with left-sided gallbladders are also important in split liver transplantation[12,25-27]. There are two ways of splitting the liver parenchyma suitable for transplantation: (1) dividing the right and left lobes of the liver into anatomical right or left lobes; and (2) separating the liver between the left medial segment and left lateral segment. During division of the right and left lobes of the liver, the portal venous pedicle is separated at the hepatic hilar region, with the left portal vein free and the right portal vein continuous with the main portal vein, or the double right portal veins free and the left portal vein continuous with the main portal vein. Hepatic venoplasty or vein graft interpositions are necessary to reconstruct these anomalous vessels. When separating the liver between the left medial and left lateral segments, the portal venous pedicle is usually divided at the hepatic hilar region, with the left portal vein free and the right portal vein continuous with the main portal vein. This conventional approach can preserve the right lobe, but the volume of left lateral segment may not be sufficient for donation from donors with a left-sided gallbladder. Preoperative evaluation of the left lateral segment volume is, therefore, critical in donors with a left-sided gallbladder.

Right anterior hepatic duct joining the left hepatic duct is a common variation in left-sided gallbladders. Its angle is too acute for possible dilatation in the treatment of the postoperative residual hepatolithiasis, with eventual stenting through the T-tube tract. Percutaneous transhepatic cholangioscopic lithotomy via the left intrahepatic duct should be considered and can provide a more favorable anatomy[24,28,29]. Left-sided gallbladders are overlapped by both the ligamentum teres and falciform ligaments, as well as by the left lobe of the liver. Although laparoscopic cholecystectomy can be performed with the standard port sites with a falciform lift, more medial positioning of the gallbladder-retracting port, and placement of the right-hand operating port to the left of the midline is suggested for laparoscopic removal of left-sided gallbladders[6,30,31].

In conclusion, left-sided gallbladders are a rare anomaly. Judicious preoperative planning is important to identify associated anomalies necessary for the safe conduct of laparoscopic cholecystectomy, hepatectomy, split liver transplantation, or living donor liver hepatectomy in transplantation. If a left-sided gallbladder is found on enhanced CT imaging, further study such as angiography, CT angiography or MRI studies can provide more detailed vascular and even biliary anomalies for preoperative planning. Although left-sided gallbladders with segment Ⅳ atrophy have been reported[5,6,8], we propose that segment Ⅳ is not atrophic but embedded in the hypoplastic right lobe. However, not all cases in our study were defined by CT and angiography.

COMMENTS

Background

Left-sided gallbladders with right-sided ligamentum teres are a rare anomaly associated portal vein anomalies, biliary system anomalies, and the absence of segment Ⅳ. The associated anomalies are important entities during liver resection, complicated hepatolithiasis, and in liver transplantation. Preoperative diagnosis of left-sided gallbladders with associated anomalies is indispensable to minimize the incidence of operative complications.

Research frontiers

Although preoperative diagnosis of left-sided gallbladders is important, there are only a few reported case series of left-sided gallbladders. We assessed the imaging presentation on US, CT and angiography of left-sided gallbladders, which was diagnosed if a right-sided ligamentum teres was present on CT.

Innovations and breakthroughs

This study of nine cases is the largest reported to date with detailed imaging presentation by US, CT and angiography. The etiology of left-sided gallbladders was investigated with the imaging presentations of hepatic vein anomalies, hepatic artery anomalies, biliary anomalies and the absence of segment Ⅳ of the liver.

Applications

Awareness of the special imaging presentations of left-sided gallbladders on US, CT and angiography is important to make an accurate diagnosis. Identification of the associated anomalies will prevent dangerous maneuvers in hepatectomy, split liver transplantation, or living donor liver hepatectomy in transplantation.

Terminology

A left-sided gallbladder is defined as a gallbladder located to the left side of the ligamentum teres. The anomalies associated with left-sided gallbladders, including

portal vein anomalies, biliary system anomalies, and segment Ⅳ atrophy, were reported. Segment Ⅳ is one of three hepatic segments that constitute the left lobe of the liver. It lies between the falciform ligament and the vertical plane of the right hepatic vein, and is demarcated on the diaphragmatic surface of the liver by a line extrapolated from the fossa for the gallbladder to the inferior vena cava; the medial segment is supplied by medial branches of the umbilical part of the left branch of the portal vein.

Peer review

The authors assessed the importance of preoperative diagnosis and presentation of left-sided gallbladders using US, CT and angiography. They concluded that left-sided gallbladders are an important clinical entity in hepatectomy, because of their associated portal venous and biliary anomalies. It should be considered in US, CT and angiography images that demonstrate no definite segment Ⅳ, absence of umbilical portion of the portal vein in the left lobe, and club-shaped right anterior portal vein.

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RAPID COMMUNICATION

New method for long-term monitoring of intragastric pH

Shouko Ono, Mototsugu Kato, Yuji Ono, Masahiro Asaka

Shouko Ono, Mototsugu Kato, Division of Endoscopy, Hokkaido University Hospital, Sapporo 060-8648, Japan
Yuji Ono, Masahiro Asaka, Department of Gastroenterology, Hokkaido University Graduate School of Medicine, Sapporo 060-8648, Japan
Correspondence to: Mototsugu Kato, Division of Endoscopy, Hokkaido University Hospital Nishi-5, Kita-14, Kita-ku, Sapporo 060-8648, Japan. m-kato@med.hokudai.ac.jp
Telephone: +81-11-7161161 Fax: +81-11-7067867
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Abstract

AIM: Consecutive monitoring of intragastric pH using the Bravo® capsule.

METHODS: We put threads through a Bravo® capsule and then affixed it to the gastric wall by endoscopic hemoclipping in seven subjects. Study data were uploaded to a computer *via* Datalink every 48 h. In this way, repeated monitoring of intragastric pH was undertaken.

RESULTS: All subjects were able to monitor gastric pH over a 1-wk period, and five for > 2 wk. No complications were encountered during the monitoring. After pH monitoring, we safely retrieved the capsule endoscopically.

CONCLUSION: Clipping a Bravo® capsule onto the gastric wall enabled long-term intragastric pH monitoring. This is a methodological report of pH monitoring over a period of > 2 wk.

Key words: Gastric pH; Ambulatory monitoring; Bravo system

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INTRODUCTION

The Bravo® pH monitoring system is a catheter-less system. It has been reported to be useful clinically because it enables monitoring without pain while the subject undertakes daily activities[1-5]. The Bravo® capsule is attached to the esophageal mucosa by suction *via* the delivery system, and it is usually eliminated spontaneously within 1 wk[2]. According to manufacturer Medtronic, the average life of a capsule's battery is 14 d, and consecutive pH monitoring is theoretically possible until the capsule battery goes flat. We used these characteristics of the Bravo® capsule to develop a new method for long-term monitoring of intragastric pH[6].

MATERIALS AND METHODS

Subjects and materials

The subjects of this study were seven *Helicobacter pylori*-negative volunteers. The Bravo® pH monitoring system (Medtronic, Shoreview, MI, USA), nylon threads, flexible overtube® (Sumitomo Bakelite, Tokyo, Japan), and stainless steel hemoclips (HX-200L-135; Olympus Optical, Tokyo, Japan) were prepared.

Preparation for Bravo® capsule

First, we removed the Bravo® capsule from its delivery system and we tied two nylon threads to the capsule and made each thread into a 2-cm diameter ring. Next, we confirmed a connection between the capsule and the receiver. The capsule was then calibrated in buffer solutions.

Placement of the Bravo® capsule

Endoscopic examinations with sedation were conducted on the subjects and results revealed the absence of any abnormal lesions. The flexible overtube was back-loaded onto the endoscope prior to the procedure. We carefully inserted the flexible overtube® with the tip of the endoscope inside the stomach. The Bravo® capsule was then passed through the tube into the stomach. Using hemoclips, the threads attached to the capsule were fixed on the greater curvature of the upper corpus (Figure 1).

Schedule

We administered antacids to determine whether the Bravo® capsule precisely monitored changes in intragastric pH. The subject was administered 150 mg ranitidine hydrochloride orally twice daily at 8:00 and 20:00 h. No limitation of meals and activities was imposed but meal times were set at 7:00, 12:00 and 19:00 h. Alcohol and tobacco were prohibited.

Analysis of pH data

Study data were uploaded to a computer *via* Datalink

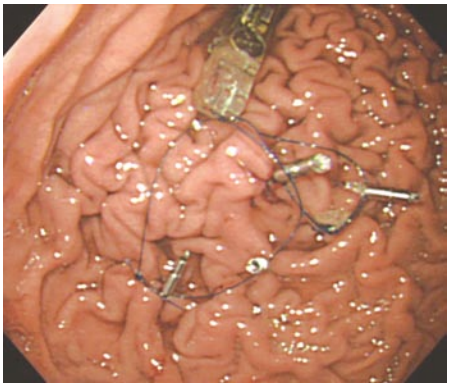

Figure 1 Bravo® capsule in the stomach. The Bravo® capsule was placed onto the gastric wall in the greater curvature of the upper corpus. Threads attached to the capsule were fixed by hemoclips.

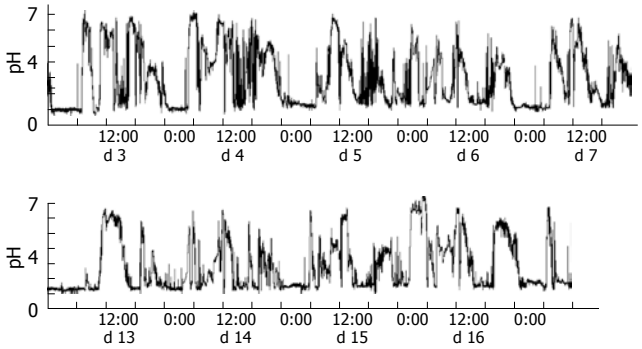

Figure 2 Intragastric pH graph monitored by the Bravo® pH capsule. The upper graph shows d 3-7 of pH monitoring, and the lower graph shows d 13-16 after placement of the Bravo® capsule in one subject.

(Medtronic) every 48 h and analyzed using basic computer software (EXCEL; Microsoft, Redmond, WA, USA). After monitoring was completed, the fixed Bravo® capsules were removed endoscopically. We cut the threads tied to the capsule using scissors forceps (FS-3L-1; Olympus Optical), and put the capsule in the Roth Retrieval Net TM (United States Endoscopy Group, Mentor, OH, USA). Then, we retrieved the capsule by pulling it into an attachment mounted on the tip of the fiberscope.

Ethics

This study was approved by our hospital ethics committee and informed consent for participation in the study was obtained from all subjects.

RESULTS

Placement of the Bravo® capsules took less than 5 min. No complications or side effects were observed during pH monitoring. All subjects were able to perform normal daily activities. Two of the seven subjects ended gastric pH monitoring because their capsule batteries went flat (at d 7 and 13). The other five succeeded in pH monitoring for 16 consecutive days. During this study, the average rate of captured data per day was 97.7%. A selection of the pH monitoring data is shown in Figure 2.

Gastric acid secretion was suppressed, beginning on the first day of ranitidine intake. However, during the medication period, the percentage time with pH < 4 increased and the median pH decreased. At the time of re-administration, the effect of the drug developed more slowly than at the first time it was given.

Retrieval of the Bravo® capsules took less than 5 min. No capsule suffered any damage.

DISCUSSION

Using the standard vacuum method, it is more difficult to attach the Bravo® capsule to a large lumen such as the stomach, than to the esophagus. It took us 15 min to place the capsule using the conventional method. The capsule was then eliminated on the fifth day. In previous studies of the Bravo® system, intragastric pH monitoring was carried out for just 48 h[7-9]. Since an endoscopic procedure and administration of premedication can influence gastric acid secretion[10], a longer period for pH monitoring would be helpful for diagnosis[11-13]. In our study, we were able to monitor gastric pH for at least 1 wk.

We had some difficulty in passing threads through the capsule beforehand, but we needed only about 5 min to affix the capsule under endoscopic guidance[7]. The Bravo® system has been reported to be safe[2,14,15], although there is a report of esophageal perforation during attachment of the capsule[16]. We used a flexible overtube® so that the capsule could pass safely through the cervical esophagus, a physiologic stricture. However, complications with the flexible overtube® have been reported[17], and so care must be taken when inserting it.

In addition, the precision of long-term pH monitoring using an antimony electrode fixed in stomach may create problems. However, in our study, pH rhythm was reflected by the effects of meals and medication, but the precision itself was not influenced. Some studies have suggested that the effects of histamine H2-receptor antagonists are attenuated during continuous treatment; an effect expressed as tolerance or tachyphylaxis[18-21]. Tachyphylaxis of ranitidine was observed in our study. The rates of captured data per day in the esophagus and stomach have been reported as 97.7% to 99.3%[7,14] and 98.3%[7], respectively. In our study, the rate was high and did not decrease over the 16-d period.

After monitoring for 16 d, we endoscopically retrieved the fixed capsules from the stomach. The Bravo® capsule attached to the esophagus by suction was eliminated spontaneously through the gastrointestinal tract. However, we confirmed that a capsule fixed to the stomach for a long period did not suffer any damage.

H2-receptor antagonists and proton-pump inhibitors rapidly and potently suppress gastric acid secretion and are widely used for treatment of acid-related diseases[22-27]. However, they have some clinical weaknesses, tachyphylaxis and nocturnal gastric acid breakthrough[28-30], which have not been resolved. One reason has been the availability of only intermittent pH monitoring, but not long-term continuous monitoring. Our method is useful clinically not only for the diagnosis of acid-related diseases, but also for the elucidation of their problems.

In conclusion, we present a newly developed method for easy and simple long-term monitoring of intragastric pH using the Bravo® pH monitoring system.

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COMMENTS

Background

The Bravo® capsule is usually attached to esophageal mucosa by suction *via* the delivery system and eliminated spontaneously within 1 wk. However, consecutive pH monitoring is theoretically possible until the capsule battery goes flat.

Research frontiers

It has been reported that an endoscopic procedure and administration of premedication can influence gastric acid secretion when using the Bravo® pH monitoring system. In previous studies of the Bravo® system using its conventional vacuum method of attachment, it was only possible to carry out intragastric pH monitoring for 48 h.

Innovations and breakthroughs

Clipping of a Bravo® capsule onto the gastric wall enabled intragastric pH monitoring for > 2 wk. The methodology is easy and simple.

Applications

Our method is clinically useful, not only for diagnosis of acid-related diseases, but also for the elucidation of tolerance of H2-receptor antagonists and nocturnal gastric acid breakthrough of proton-pump inhibitors.

Terminology

Bravo® pH monitoring system is a catheter-less pH monitoring system.

Peer review

The authors have reported a modified method for long-term monitoring of intragastric pH using the Bravo capsule fixed on the gastric wall by endoscopic hemoclipping. The contents of the manuscript are reasonable, and this may be a useful method for the elucidation of tolerance to H2-receptor antagonists and nocturnal gastric acid breakthrough of proton-pump inhibitors, as the authors state.

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RAPID COMMUNICATION

Personality factors and profiles in variants of irritable bowel syndrome

Alireza Farnam, Mohammad H Somi, Firouz Sarami, Sara Farhang, Sanaz Yasrebinia

Alireza Farnam, Mohammad H Somi, Firouz Sarami, Sara Farhang, Sanaz Yasrebinia, Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
Correspondence to: Sara Farhang, Liver and Gastrointestinal Diseases Research Center, Imam Hospital, University Ave, Tabriz, East Azerbaijan, Iran. sfarhang@yahoo.com
Telephone: +98-411-3367473 Fax: +98-411-3367499
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Abstract

AIM: To study the association between irritable bowel syndrome (IBS) variants (constipation, diarrhea, or both) and personality traits in non-psychiatric patients.

METHODS: IBS was diagnosed using the Rome Ⅱ diagnostic criteria after exclusion of organic bowel pathology. The entry of each patient was confirmed following a psychiatric interview. Personality traits and the score of each factor were evaluated using the NEO Five Factor Inventory.

RESULTS: One hundred and fifty patients were studied. The mean age (± SD) was 33.4 (± 11.0) year (62% female). Subjects scored higher in neuroticism (26.25 ± 7.80 *vs* 22.92 ± 9.54, $P < 0.0005$), openness (26.25 ± 5.22 *vs* 27.94 ± 4.87, $P < 0.0005$) and conscientiousness (32.90 ± 7.80 *vs* 31.62 ± 5.64, $P < 0.01$) compared to our general population derived from universities of Iran. Our studied population consisted of 71 patients with Diarrhea dominant-IBS, 33 with Constipation dominant-IBS and 46 with Altering type-IBS. Scores of conscientiousness and neuroticism were significantly higher in C-IBS compared to D-IBS and A-IBS (35.79 ± 5.65 *vs* 31.95 ± 6.80, $P = 0.035$ and 31.97 ± 9.87, $P = 0.043$, respectively). Conscientiousness was the highest dimension of personality in each of the variants. Patients with C-IBS had almost similar personality profiles, composed of higher scores for neuroticism and conscientiousness, with low levels of agreeableness, openness and extraversion that were close to those of the general population.

CONCLUSION: Differences were observed between IBS patients and the general population, as well as between IBS subtypes, in terms of personality factors. Patients with constipation-predominant IBS showed similar personality profiles. Patients with each subtype of IBS may benefit from psychological interventions, which can be focused considering the characteristics of each subtype.

Key words: Irritable bowel syndrome; Personality; Conscientiousness; Neuroticism; Openness; Constipation-predominant

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder with a wide variety of presentations that include abdominal pain, bloating, disturbed defecation (constipation and/or diarrhea) or alternating bowel habits, and the absence of any detectable organic pathological process[1]. Symptom-based criteria along with limited medical evaluation are used for diagnosis. Treatment is challenging because of the heterogeneity of the presenting symptoms, together with the unclear pathophysiology of the disorder. To date, treatment strategies are focused on specific symptoms, potential underlying disorders in stress responsiveness, and predisposing psychological features.

Regardless of the unclear etiology of the syndrome, it is commonly accepted as being a disorder closely influenced by affective factors. IBS seems to be influenced by psychosocial stressors and psychiatric comorbidity. The incidence of mood and anxiety disorders has been well studied in IBS patients. The high rates of psychiatric co-morbidity in IBS patients indicate that the affective symptoms may be specific and integral to the syndrome, rather than be a specific syndrome related to a chronic intestinal disease[2]. This has resulted in recommendations on how to best detect and integrate treatments to achieve better outcomes for these patients[3], and has led to significant improvements. Patients and physicians might benefit from detailed identification and psychotherapeutic intervention in patients with comorbid psychiatric disorders (as opposed to psychodynamic personality dysfunction).

However, the underlying personality structure might be misinterpreted at the present time because of the

poor quality and dubious results of early research done in the area of personality factors as they relate to IBS[4]. The five-factor model of personality defines personality traits in terms of five basic dimensions: extraversion, which incorporates talkativeness, assertiveness and activity level; agreeableness, which includes kindness, trust and warmth; conscientiousness, which includes organization, thoroughness and reliability; neuroticism versus emotional stability, which includes nervousness, moodiness and temperamentality; and openness to experience, which incorporates imagination, curiosity and creativity. This model has been widely accepted because the structure of traits in it is consistent among highly diverse cultures with various languages, and between men and women, and older and younger adults[5].

Patients are often subclassified by their predominant bowel habits, that is, constipation-predominant, diarrhea-predominant, or alternating diarrhea and constipation. Patients with IBS share basic pathophysiological features, regardless of bowel habits; however, differences in perception, autonomic function, and symptom characteristics between constipation-predominant and diarrhea-predominant patients have been described[6-8]. Psychological treatment has been reported to be more effective for diarrhea- than constipation-predominant patients[9]. The association between psychological features and specific symptoms of IBS has been minimally explored. We hypothesized that personality traits are also associated with the dominant symptom of IBS. We sought to assess the distribution of personality traits in IBS patients in our cohort as a first step, and then define any relationship with dominant symptoms.

MATERIALS AND METHODS

Continuous patients attending our university outpatient clinics with a diagnosis of IBS were included. Diagnosis was established after a stool examination, clinical evaluation and endoscopy (in some cases) by a gastroenterologist using the ROME Ⅱ criteria for IBS. All patients were clinically investigated to identify the presence of "alarm factors". Patients were excluded if an organic cause of the condition were possible, or if there were a history of serious somatic disease. All patients gave informed written consent. Demographic information, severity and course of illness, abdominal pain severity over previous weeks, bowel habits and gastrointestinal symptoms were obtained. Patients described whether their symptoms arose with stress and if/how they disrupted their daily activities. Patients were divided into three groups: constipation-predominant (C-IBS), diarrhea-predominant (D-IBS) and altering diarrhea or constipation (A-IBS), according to their self-explanation of recent symptoms.

Patients were referred to the first author, who was blinded to characteristics of IBS, for psychiatric and psychosomatic assessments. A history or current symptoms of any DSM-IV psychiatric diagnosis[11] on axis Ⅰ or seizure disorders led to exclusion from the study.

Personality dimensions in non- psychiatric IBS patients were evaluated by the NEO Five-Factor Inventory (NEO-FFI), a 60-item questionnaire which usually requires 10-15 min to complete. This questionnaire is rated on a five-point scale to yield scores in five major domains of personality and requires a sixth-grade reading level. Scores of five personality factors measured by NEO have previously been described in a survey of an Iranian population of all universities[10].

Table 1 Mean (SD) scores of five personality factors measured by FFI in patients with irritable bowel syndrome compared to the Iranian general population

	IBS patients	General population	*P*
Openness	26.25 (5.22)	27.94 (4.87)	< 0.0005
Conscientiousness	32.90 (7.80)	31.62 (5.64)	< 0.0005
Extraversion	27.06 (6.09)	26.89 (6.15)	0.733
Agreeableness	28.97 (6.74)	32.90 (7.00)	0.344
Neuroticism	26.25 (7.80)	22.92 (9.54)	< 0.0005

Statistical analysis

Data were analyzed using the SPSS Statistical package ver.13 (SPSS, Chicago, IL, USA). Means ± SD were used to describe continuous variables and proportions for categorical data. Conditions were met for using two-tailed Student's t test and Chi-square test, which were applied when appropriate. Within- and between-group comparisons were performed using ANOVA. The Bonferroni inequality test was used to affirm that significance was not reached by chance alone. Overall significance was set at 0.05.

RESULTS

One hundred and fifty patients age, mean ± SD was 33.4 ± 11.0 year (62% female), with IBS by ROME Ⅱ criteria were enrolled in the study. Bowel problems that were provoked by distress in > 80% of patients interrupted daily activities in up to 70%.

NEO-FFI showed a significantly higher level of neuroticism and conscientiousness and a lower level of openness in the non-psychiatric IBS patients. Table 1 shows mean scores for five personality factors in our patients compared to the Iranian general population (by NEO FFI)[12]. Women with IBS had significantly higher levels of neuroticism, conscientiousness and extraversion compared to men (P = 0.032, 0.003 and 0.037 in that order) (Figure 1).

Our study population consisted of 71 patients with D-IBS, 33 with C-IBS and 46 with A-IBS. Patient occupation, educational level and marriage status had similar patterns among the groups. Symptoms reported by patients with C-IBS and A-IBS were more related to stressors (P = 0.004).

The score for conscientiousness was significantly higher in C-IBS (35.79 ± 5.65) than D-IBS (31.95 ± 6.80) and A-IBS (31.97 ± 9.87) (P = 0.035 and 0.043). Neuroticism had a higher score in C-IBS compared to the other groups (P = 0.044). Conscientiousness was the highest dimension of personality in each of the variants: 42% of C-IBS, 55% of D-IBS and 47% of A-IBS patients.

Personality profiles were somewhat capricious in

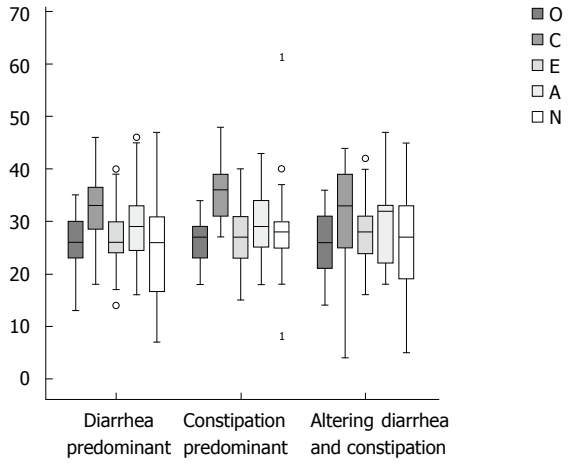

Figure 1 Scores of personality factors in irritable bowel syndrome patients, defined by dominant symptom. Box plots show distribution of the scores, defining means, minimum, maximum, range, interquartile range and cases out of 95% confidence intervals(as[1]). O: Openness to Experience; C: Conscientiousness; E: Extraversion; A: Agreeableness; N: Neuroticism.

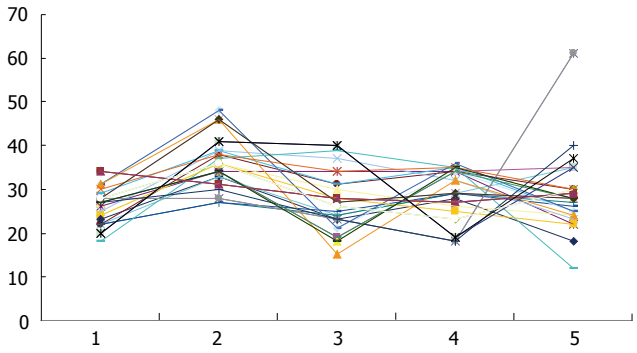

Figure 2 Personality profiles in patients with C-IBS. 1: Openness to Experience; 2: Conscientiousness; 3: Extraversion; 4: Agreeableness; 5: Neuroticism.

patients with A-IBS and D-IBS. Whereas, patients with C-IBS had mostly similar personality profiles (Figure 2), which showed higher scores for neuroticism and conscientiousness, a low level of agreeableness, along with openness and extraversion close to those of the general population.

DISCUSSION

The present study was designed to investigate personality characteristics of non-psychiatric IBS patients considering their IBS subtype. Emotional states and personality traits may affect the physiology of the gut[13], and play a role in how symptoms are experienced and interpreted, and can thus influence treatment[14,15]. This can be an important issue when considering a management strategy to achieve a better outcome for an IBS patient. The prevalence of IBS has been reported to be 18.4% in the general Iranian population[16].

The five-factor model provides a dimensional account of the structure of normal personality traits, dividing personality into five broad dimensions. There has been little research examining the biological correlates of the dimensions and very little in known about the personality structure in IBS patients. Neuroticism and aggression are reported to be higher in patients with functional gastrointestinal disease without psychiatric comorbidity, and personality traits are believed to influence pain reporting[17]. A low level of neuroticism and little concealed aggressiveness is reported to predict treatment outcome with antidepressants in non-psychiatric patients, which are most prominent in women. These personality dimensions are better predictors of outcome than serotonergic sensitivity[18].

Differences between male and female patients with IBS have been reported; the significant differences found here in the traits of neuroticism, extraversion and conscientiousness were consistent with other studies that suggest women consistently score higher than men on self-reported trait anxiety[19,20]. The data for non-psychiatric individuals drawn from a pool prepared for standardization of the Iranian version of NEO PI-R are among the limitations of the present study. For a more appropriate comparison, the control individuals in future studies might be beneficially selected from the member of patients' family.

Studies on personality dimensions according to subtypes of IBS are limited. Similar personality dimensions (by the Minnesota Multiphasic Personality Inventory) have been reported in subgroups of IBS patients with predominant constipation and for those with predominant diarrhea[21].

In the current study, C-IBS patients scored higher on neuroticism and conscientiousness. Neuroticism is a personality trait characterized by overstated reactivity to physiological changes. According to Costa and McCrae[5], people with elevated scores on the neuroticism dimension are emotionally unstable with overwhelmingly negative emotions. Neuroticism is related to emotional intelligence, which involves emotional regulation, motivation, and interpersonal skills. Hans Eysneck theorized that neuroticism is a function of activity in the limbic system, and research suggests that people who score high on neuroticism have a more reactive sympathetic nervous system, and are more sensitive to environmental stimulation[22]. Centrally targeted medications, such as anxiolytics and low-dose tricyclic antidepressants, which involve inhibitory effects on the sensitivity of emotional motor system[23] are widely used for C-IBS patients[24] as they can balance the neuroticism dimension of such patients.

Individuals with high self-consciousness are not at ease with others, are sensitive to ridicule, and prone to feelings of inferiority. This is compatible with a lack of success in completing the "anal stage" of Freud's theory of psychosexual development, which is supposed to result in an obsession with perfection and control. Such patients may benefit from selective serotonin re-uptake inhibitors which increase the extracellular level of the neurotransmitter serotonin.

Personality disorders can be conceptualized as extreme variants of the normal personality dimensions[25]. Excluding such disorders, we sought the personality make up that can introduce vulnerable profiles for IBS in a cross-sectional manner. Research has focused on incorporating various forms of psychotherapy in the hope of alleviating symptoms. Psychological interventions

have been aimed at individuals and at groups and include insight-oriented psychotherapy, hypnotherapy, behavior therapy and group psychotherapy. Such interventions try to address various unconscious conflicts in the subject and thereby help them re-establish a sense of emotional stability[26], modify maladaptive behavior and seek new solutions to problems[27], incorporate multi-component cognitive-behavioral therapy treatment programs[28], and have reported significant improvement in symptoms. It is felt that the profession of counseling psychology, which looks to develop wellness, strength and resources within individuals, has the potential to make a unique contribution to the prevention and alleviation of IBS[29]. Presently, the effectiveness of psychological treatments in IBS is being reviewed[30] in the light of conflicting evidence that supports the use of psychological treatment, an inadequate methodology for randomized controlled trials in this area, and the limited evidence of how to improve the global health of IBS patients with drug therapy[31]. The present study presents added evidence of the differences between subgroups of IBS. As such, this may help to focus management plans in each subgroup to obtain better outcomes.

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COMMENTS

Background

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder that is commonly accepted as a disorder closely related to psychological factors. Patients might benefit from detailed identification and psychotherapeutic interventions.

Research frontiers

Mood and anxiety disorders are well studied in IBS patients but information about any underlying personality structure is lacking. Additionally, differences in subtypes of IBS (according to dominant symptom) have been described in perception, autonomic function, and symptom characteristics, which are recommended for evaluation in IBS patients according to their presenting symptoms. This report elucidates personality characteristics of IBS patients according to their dominant symptom using a well-designed personality questionnaire.

Innovations and breakthroughs

IBS patients scored higher on neuroticism, openness and conscientiousness compared to the general Iranian population. Conscientiousness was the highest dimension of personality in each of the variants. The scores for conscientiousness and neuroticism were significantly higher in C-IBS compared to D-IBS and A-IBS. An analogous personality profile was noted in patients with C-IBS composed of the higher scores of neuroticism and conscientiousness, a low level of agreeableness, and with openness and extraversion similar to those of the general population.

Applications

Overstated personality dimensions of IBS patients (especially the constipation-dominant subtype) can be balanced by the use of well-known medications, like anxiolytics, low-dose tricyclic antidepressants and selective serotonin re-uptake inhibitors. Given the added evidence of the differences between subtypes of IBS and vulnerable personality profiles, future studies on drug or psychotherapy may achieve more reliable outcomes.

Terminology

IBS is a gastrointestinal disorder that has a wide variety of presentations that include abdominal pain, bloating, disturbed defecation (constipation and/or diarrhea or alternating bowel habits) and the absence of any detectable organic pathological process. NEO-FFI is a questionnaire with a five-point scale to yield scores in five major domains of personality: openness to experience, conscientiousness, extraversion, agreeableness and neuroticism

Peer review

The results and conclusions of the paper are reliable. The presentation is adequate and easy to read. There are no ethics problems. This is a good manuscript with an interesting approach to the subject.

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RAPID COMMUNICATION

Prevalence of fatty liver disease and its risk factors in the population of South China

Yong-Jian Zhou, Yu-Yuan Li, Yu-Qiang Nie, Jin-Xiang Ma, Lun-Gen Lu, Sheng-Li Shi, Min-Hu Chen, Pin-Jin Hu

Yong-Jian Zhou, Yu-Yuan Li, Min-Hu Chen, Pin-Jin Hu, Department of Gastroenterology, The First Affiliated Hospital, Sun Yat-Sen University of Medical Sciences, Guangzhou 510080, Guangdong Province , China
Yong-Jian Zhou, Yu-Yuan Li, Yu-Qiang Nie, Sheng-Li Shi, Department of Gastroenterology and Hepatology, First Municipal People's Hospital of Guangzhou, Guangzhou Medical College, Guangzhou 510180, Guangdong Province, China
Jin-Xiang Ma, Department of Prevention Medicine, Guangzhou Medical College, Guangzhou 510182, Guangdong Province, China
Lun-Gen Lu, Department of Gastroenterology, Renji Hospital, Shanghai Institute of Digestive Disease, Shanghai Jiaotong University School of Medicine, Shanghai 200001, China
Supported by a Grant from Guangzhou Health Bureau Project, No. 2004-Z001
Correspondence to: Yu-Yuan Li, Department of Gastroenterology and Hepatology, First Municipal People's Hospital of Guangzhou, No. 1 Panfu Road, Guangzhou 510180, Guangdong Province, China. liyyliyy@tom.com
Telephone: +86-20-81048720 Fax: +86-20-81045937
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Abstract

AIM: To investigate the population-based prevalence of fatty liver disease (FLD) and its risk factors in Guangdong Province, China.

METHODS: A cross-sectional survey with multiple-stage stratified cluster and random sampling of inhabitants over 7-year-old was performed in 6 urban and rural areas of Guangdong Province, China. Questionnaires, designed by co-working of epidemiologists and hepatologists, included demographic characteristics, current medication use, medical history and health-relevant behaviors, i.e., alcohol consumption, smoking habits, dietary habits and physical activities. Anthropometric measurements, biochemical tests and abdominal ultrasonography were carried out.

RESULTS: Among the 3543 subjects, 609 (17.2%) were diagnosed having FLD (18.0% males, 16.7% females, $P > 0.05$). Among them, the prevalence of confirmed alcoholic liver disease (ALD), suspected ALD and nonalcoholic fatty liver disease (NAFLD) were 0.4%, 1.8%, and 15.0%, respectively. The prevalence rate (23.0%) was significantly higher in urban areas than (12.9%) in rural areas. After adjustment for age, gender and residency, the standardized prevalence of FLD in adults was 14.5%. Among them, confirmed ALD, suspected ALD and NAFLD were 0.5%, 2.3%, and 11.7%, respectively, in adults and 1.3% (all NAFLD) in children at the age of 7-18 years. The overall prevalence of FLD increased with age in both genders to the peak of 27.4% in the group of subjects at the age of 60-70 years. The prevalence rate was significantly higher in men than in women under the age of 50 years (22.4% *vs* 7.1%, $P < 0.001$). However, the opposite phenomenon was found over the age of 50 years (20.6% *vs* 27.6%, $P < 0.05$). Multivariate and logistic regression analysis indicated that male gender, urban residency, low education, high blood pressure, body mass index, waist circumference, waist to hip ratio, serum triglyceride and glucose levels were the risk factors for FLD.

CONCLUSION: FLD, especially NAFLD, is prevalent in South China. There are many risk factors for FLD.

Key words: Fatty liver disease; Prevalence; Epidemiology; Risk factors

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INTRODUCTION

Fatty liver disease (FLD), defined as lipid accumulation exceeding the normal range of 5% of liver wet weight, is a chronic disorder, which includes alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD). FLD encompasses a morphological spectrum consisting of hepatic steatosis and steatohepatitis which can progress to cirrhosis and hepatocellular carcinoma[1]. In the past, excess alcohol consumption accounted for most cases of FLD, but recently nonalcoholic causes of fatty liver have attracted considerable attention. In addition to alcohol consumption, factors such as insulin resistance (IR), oxidative stress, mitochondrial dysfunction, immune deregulation, and adipokines play an important role in the pathogenesis of FLD[2,3].

FLD has become a common problem in both developed and developing countries. According to the

Office for National Statistics in the United Kingdom, liver disease mainly FLD ranks the fifth most common cause of death after heart disease, stroke, pulmonary disease and cancer[4]. In recent years, due to alterations in life style and dietary habits, the incidence of FLD has increased dramatically in China. It is thus of importance to assess the epidemiological features of FLD in this country in order to facilitate its prevention and treatment. Although some epidemiological studies concerning FLD have been reported in China, most of them were based on clinical settings or on health check-up groups, which do not represent population data. So far only one population-based study from Shanghai, an eastern city of China, has been published in English[5]. The data from rural areas and other parts of this country are still lacking.

In this study, we investigated the prevalence of FLD in population of South China and identified its major risk factors. Since the random sampling method and high response rate limited the selection and responder biases, our results reflect the population-based data in both urban and rural areas.

MATERIALS AND METHODS

Study design

Guangdong Province with a population of about 85 millions (mainly Han nationality people) is located in an economically advanced region in South China. Its capital city, Guangzhou, has 8-million residents. The heterogeneity of economy, geography, and culture in different areas inside the province influences the life styles of the people.

Between April and November of 2005, a cross-sectional survey with multiple-stage stratified cluster and random sampling was performed on the basis of a previous analysis of the population distribution in this province[6,7]. In order to represent the data in general population, sampling clusters were randomly drawn from urban and rural areas all over the province. Six areas with 4365 residents over 7 years of age were selected, i.e., 2 communities from downtown of Guangzhou city (Yuanzhongyuan community of the Liwan district and the Xinyi community of the Baiyun district, with a total of 1436 urban residents) and four units within the province, i.e., a village in central area of the province (Huadu county, 822 rural residents), a village in southern area (Huizhou county, 1037 rural residents), a village in western area (Zhanjiang county, 351 rural residents) and a community in northern area (Shaoguan city, 719 urban residents).

Of the 4365 potential participants, 3903 agreed to take part in this investigation with a responder rate of 89.6%. Among them, 3543 subjects (90.6%, 1311 males and 2232 females, at the age of 7-100 years) provided their complete information, accounting for approximately 44.3/100 000 of the total population of Guangdong Province[7]. Interview, physical examination, laboratory assessment and ultrasonographic examination were performed for each subject on the same day at a mobile examination center. The study was approved and supported by Guangzhou Health Bureau. Written consent was obtained from each participant.

Interview and physical examination

A face-to-face interview was carried out by specially trained post-graduate students of Guangzhou Medical College and supervised by experienced investigators. Standard questionnaires, designed by co-working of epidemiologists and hepatologists, included the following items: demographic characteristics, current medication use, medical history and health-relevant behaviors, i.e., alcohol consumption, smoking habits, dietary habits and physical activities. Physical examination covered body height, weight, blood pressure, waist circumference (WC), waist-to-hip ratio (WHR) and routine anthropometric parameters in healthy check-up.

Biochemical tests

Intravenous blood samples were collected from fasting subjects by routine methods. Fasting serum glucose levels and lipid profiles were measured with an automatic chemistry- immuno-analyzer (Olympus Corporation, Tokyo, Japan). Serum asparatate aminotransferase (AST), and alanine aminotransferase (ALT), serum bilirubin (BIL) and albumin levels were measured by standard laboratory methods. Tests for the serum makers of hepatitis A virus (HAV), hepatitis B virus (HBV) and hepatitis C virus (HCV) were also carried out.

Ultrasonography

Real-time ultrasonography (US) of the upper abdominal organs was performed for each subject by 2 experienced physicians using a scanner equipped with a 3.5 mmHz transducer (Siemens Adama, German). The physicians performing the US were unaware of the clinical and laboratory results.

Diagnostic criteria

FLD, NAFLD and ALD were diagnosed according to the guidelines for diagnosis and treatment of nonalcoholoic and alcoholoic fatty liver diseases issued by Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association[8,9], which were adapted from the American Gastroenterological Association[10]. Briefly, the diagnosis of FLD, NAFLD and ALD was based on the combination of medical history, clinical symptoms, laboratory and US findings. Viral hepatitis and other chronic liver diseases needed to be ruled out. Liver biopsy was taken when the diagnosis was suspected. In this epidemiological study, only 12 subjects underwent biopsy. In most cases FLD was diagnosed and staged by US findings. ALD was diagnosed when a subject fulfilling the FLD criteria drank more than 40 g (male) or 20 g (female) alcohol per day over 5 years. Suspected ALD was defined when the alcohol consumption was 20-40 g (male) or 10-20 g (female). NAFLD was diagnosed when the alcohol consumption was less than these amounts.

The US diagnostic patterns of FLD were based on the presence of a 'bright' liver (brightness and posterior attenuation) with stronger echoes in the hepatic parenchyma than in the renal parenchyma, vessel blurring and narrowing of the lumen of hepatic veins in the absence of findings suggestive of other chronic liver

Table 1 Age and sex-specific prevalence of fatty liver disease

	Total		Male		Female		*P*
	n	*n* (%)	*n*	*n* (%)	*n*	*n* (%)	
7-	379	5 (1.3)	210	4 (1.9)	169	1 (0.6)	0.265
18-	349	20 (5.7)	129	14 (10.9)	220	6 (2.7)	0.002
30-	477	53 (11.1)	155	31 (20.0)	322	22 (6.8)	0.000
40-	667	101 (15.1)	193	46 (23.8)	474	55 (11.6)	0.000
50-	809	214 (26.5)	230	53 (23.0)	579	161 (27.8)	0.166
60-	551	151 (27.4)	277	65 (23.5)	274	86 (31.4)	0.037
70-	311	65 (20.9)	117	23 (19.7)	194	42 (21.6)	0.676
Total	3543	609 (17.2)	1311	236 (18.0)	2232	373 (16.7)	0.326

Table 2 Sex-specific prevalence in fatty liver disease subgroups

		Confirmed ALD	Suspected ALD	NAFLD	Total (FLD)
	n	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Male	1311	12 (0.9)	53 (4.0)	171 (13.1)	236 (18.0)
Female	2232	2 (0.1)	12 (0.5)	359 (16.1)	373 (16.7)
Total	3543	14 (0.4)	65 (1.8)	530 (15.0)	609 (17.2)
P		< 0.001	< 0.001	< 0.05	> 0.05

FLD: Fatty liver disease; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

Table 3 Residence-specific prevalence in FLD subgroups

		Confirmed ALD	Suspected ALD	NAFLD	Total (FLD)
	n	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Rural	2047	11 (0.5)	27 (1.3)	227 (11.1)	265 (12.9)
Urban	1496	3 (0.2)	38 (2.5)	303 (20.3)	344 (23.0)
Total	3543	14 (0.4)	65 (1.8)	530 (15.0)	609 (17.2)
P					< 0.001

FLD: Fatty liver disease; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

diseases. The degree of FLD evaluated by US reflected the degree of steatosis. A liver displaying increased but homogenous dot reflection, blurred but still discernible vascular profiles and weak portal vein echogenicity was defined as early-stage. A medium-stage FLD was characterized by disappearance of the portal vascular wall and slightly increased liver volume. Patients with nebulous optical appearance in the liver, little portal vein echogenicity in more than 1/3-1/2 of the liver area and increased liver volume were considered to be in the advanced stage[11-13].

Obesity was categorized according to the body mass index (BMI) criteria for Asians issued by the Regional Office for Western Pacific Region of the World Health Organization[14]. Subjects with BMI ⩾ 25 were considered as obese, BMI ⩾ 23 but < 25 as overweight, BMI ⩾ 18.5 but < 23 as normal, BMI < 18.5 as underweight. Central (or abdominal) obesity was estimated by waist circumference (WC) and waist-to-hip ratio (WHR). A WHR value ⩾ 0.9 (male) or ⩾ 0.8 (female) was considered as central obesity. Dyslipidemia was considered when having one of following serum lipid profiles: total cholesterol (TC) ⩾ 5.72 mmol/L, triglycerides (TG) ⩾ 1.70 mmol/L, high-density lipoprotein cholesterol (HDL-C) < 0.91 mmol/L, and low-density lipoprotein cholesterol (LDL-C) ⩾ 3.64 mmol/L. Subjects with their fasting serum glucose (FSG) value of ⩾ 7.0 mmol/L and/or with a history of diabetes were considered to have diabetes mellitus. Hypertension was defined as systolic or diastolic blood pressure above 140 mmHg or 90 mmHg, respectively. Smokers in this study were defined as those smoking more than one cigarette each day for at least one year or more than 20 packages of cigarettes in total. Alcohol drinkers were defined as those drinking more than 2 times each month for more than 5 years. Heavy tastes meant the habits of preferring salted and spiced foods. Extraverted character meant the active personal characters of subjects. The definitions of urban and rural areas were in accordance with the publication of the Fifth Chinese National Population Census in 2000[7].

Statistical analysis

Data were examined with the SPSS 13.0 for Windows. $P < 0.05$ was considered statistically significant. Exposure ratio comparison and multivariate regression analyses were performed to evaluate the risk factors.

RESULTS

Age and gender-specific prevalence of FLD

Data are shown in Tables 1 and 2. Of the 3543 subjects, 609 (17.2%) were diagnosed having FLD, among them 367 (10.4%), 218 (6.1%) and 24 (0.7%) fell into early, medium and advanced degrees by US staging. The overall prevalence of FLD increased to the peak of 27.4% in the group at the age of 60- years and then decreased in both genders ($P < 0.01$). There was no prevalent difference between males and females (18.0% *vs* 16.7%, $P > 0.05$). However, FLD was more common in males than in females under 50 years of age. An opposite trend was noted over 50 years of age ($P < 0.05$). The prevalence of FLD in children at the age of 7-18 years (5/379, 1.3%) differed significantly from that in adults (604/3164, 19.1%, $P < 0.001$). The overall prevalence of ALD, suspected ALD and NAFLD was 0.4%, 1.8% and 15%, respectively, accounting for 2.3%, 10.7% and 87.0% of the 609 FLD subjects, respectively. In subgroup comparison, the prevalence of confirmed ALD and suspected ALD was significantly higher in males (0.9% and 4.0%) than in females (0.1% and 0.5%, $P < 0.001$). Nevertheless, the prevalence of NAFLD was lower in males than in females (13.0% *vs* 16.1%, $P < 0.05$).

Prevalence of FLD in urban and rural areas

The prevalence of FLD was significantly lower in rural areas than in urban areas (12.9% *vs* 23.0%, $P < 0.001$), mainly due to the difference in NAFLD (11.1% *vs* 20.3%, $P < 0.001$). A slight difference was observed in rural areas between ALD and suspected ALD subjects compared with urban areas, but it was not significant ($P > 0.05$) (Table 3).

Prevalence of FLD and serum lipid profiles

High serum levels of TG, TC, LDL-C and FSG were all positively associated with FLD ($P < 0.01$), but HDL-C did not reach significance ($P > 0.05$) (Table 4). The prevalence of FLD in dyslipidemia subjects was 3.65 (95% CI = 2.87-4.65) folds higher than that in normal controls. High TG levels were the most prominent factor for dyslipidemia

Table 4 Serum levels and lipid profiles in FLD patients and controls (mean ± SE)

	n	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	FSG (mmol/L)
FLD	609	2.81 ± 2.55	5.52 ± 1.11	1.72 ± 1.25	3.06 ± 0.91	6.01 ± 2.68
Controls	2934	1.63 ± 1.59	5.19 ± 1.07	1.67 ± 0.57	2.90 ± 0.81	4.95 ± 1.56
P		< 0.001	< 0.01	> 0.05	< 0.01	< 0.01

FLD: Fatty liver disease; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

Table 5 Exposure ratios of variables in adult FLD patients compared with controls

n	Male	Urban	Primary school and below	Extraverted character	Alcohol drinkers	Smokers
Total 3164	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
FLD 604	232 (38.4[1])	344 (57.0)	245 (40.6)	214 (35.4[a])	141 (23.3)	160 (26.5[a])
Controls 2560	869 (33.9)	1140 (44.5)	783 (30.6)	787 (30.7)	457 (17.9)	553 (21.6)
χ^2	4.295	30.282	22.112	4.966	9.619	6.665
n	Heavy tastes	Overweight	Obesity	Hypertension	WHR	WC
Total 3164	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
FLD 604	208 (34.4)	478 (79.4)	120 (19.9)	287 (47.6)	531 (87.9)	432 (71.5)
Controls 2560	689 (26.9)	650 (25.5)	79 (3.1)	658 (25.7)	1469 (57.8)	558 (21.8)
χ^2	13.616	616.708	235.812	110.141	189.387	560.357

[a]$P < 0.05$ *vs* controls and all the others; $P < 0.01$ *vs* controls. FLD: Fatty liver disease; WC: Waist circumference; WHR: Waist-to-hip ratio.

with an odd ratio (OR) of 4.49 (95% CI = 3.56-5.66). The OR of high TC was 1.95 (95% CI = 1.56-2.44). The OR of elevated FSG and combined dyslipidemia was 4.93 (95% CI = 3.55-6.84) and 6.64 (95% CI = 4.83-9.12), respectively.

Adjusted prevalence of FLD

After adjustment for gender, age and residence area according to the demographic characteristics of the Fifth Chinese Census[7], the age-, gender- and residency- specific standardized prevalence of FLD was 14.5%. Confirmed ALD, suspected ALD and NAFLD were 0.5%, 2.3% and 11.7% respectively in adults (over 18 years of age). The overall prevalence of FLD was 11.3% and confirmed ALD, suspected ALD and NAFLD were 0.4%, 1.8% and 9.1% respectively, in all people over 7 years of age.

Exposure ratio and logistic regression analysis

The exposure ratio (%) analysis of anthropometric and clinical parameters in adults (above 18 years of age, $n = 3164$) with and without FLD was carried out to screen for the relevant factors for FLD (Table 5). The ratios of all variables for FLD and controls were significantly different ($P < 0.01$). In order to identify the risk factors, we further performed a multivariate regression logistic analysis with probability for entry 0.05 and removal 0.1 (Table 6). Male gender, urban inhabitation, hypertension, high BMI, WC, WHR, serum TG, and FSG were found to be independent risk factors for FLD, and education was a protective factor for FLD. Alcoholic consumption and heavy tastes in food were marginally but not significantly related to FLD. Smoking and extraverted character were excluded from the risk factors for FLD ($P > 0.05$).

Table 6 Multivariate regression logistic analysis for FLD ($n = 3543$)

Variables	β	SE	χ^2	*P*	OR	OR 95%CI
Male	0.628	0.218	5.361	0.002	0.765	0.321-0.921
Urban	0.212	0.128	28.219	< 0.001	0.332	0.211-0.291
Education level	-0.351	0.105	11.124	0.001	0.704	0.573-0.865
Hypertension	0.362	0.158	5.277	0.022	1.436	1.055-1.957
BMI	1.234	0.130	90.488	< 0.001	3.435	2.664-4.430
WHR	0.811	0.228	12.648	< 0.001	2.250	1.439-3.518
WC	1.017	0.174	33.987	< 0.001	2.765	1.964-3.892
TG	0.623	0.158	15.535	< 0.001	1.864	1.367-2.540
FSG	0.788	0.129	37.187	< 0.001	2.200	1.708-2.835
Alcohol-drinking	0.373	0.212	3.112	0.078	1.452	0.959-2.198
Heavy tastes	0.307	0.169	3.286	0.070	1.360	0.975-1.895

FLD: Fatty liver disease; BMI: Body mass index; WHR: w-to-hip ratio; WC: Waist circumference; TC: Total cholesterol; FSG: Fasting serum glucose.

DISCUSSION

FLD is a common disease. NAFLD has been increasingly recognized as the most common liver disease in Western countries. So far no accurate incidence is available. The prevalent data obtained from clinical series and autopsy studies suggest that 20%-30% of individuals in the Western world have FLD. In general population, the prevalence of NAFLD ranges 3%-24% in the world[15-17], 20%-25% in Italy[18], 30% in Israel[19], 16% in Korea[20], 14% in Japan[21] and 15% (18% for FLD) in Shanghai, China[25]. The discrepancy among the studies is probably due to the methods of sample selection, modalities used for diagnosis and diversity of life styles and dietary habits in different areas. Since China is a developing country, it is not surprising that the standardized prevalence of FLD (14.5%) and NAFLD (11.7%) is lower in China than in developed countries. The urban prevalence is lower in Guangdong Province than in Shanghai city[5]. It seems that the rapid modernization of Shanghai has brought about its inhabitants more metabolic problems than that of Guangdong Province. Our results in adults and children are comparatively close to those in Japan, probably because the inhabitants in South China share the similar dietary habits (sea food preference) with Japanese[21]. In this study, only 24 cases (0.7% in general population) had advanced stage of FLD diagnosed by US, accounting for 3.9% of the 609 FLD cases. Although US is reasonably accurate as compared with biopsy, it cannot provide precise information about the histological features associated with disease progression especially inflammation[15]. The results in this study roughly reflect the lower severity of FLD in this area.

There are conflicting results regarding the relation of NAFLD to age and gender[15-18]. Bedogni *et al*[18] found that the prevalence of NAFLD increases with age in both genders and then significantly decreases over 66 years of age[18]. However, it was reported that hepatic triglyceride is correlated with age only in white women[16]. The variations between studies can be attributed to the differences in social, cultural and environmental backgrounds among the target subjects. In this study, the highest prevalence of FLD (mainly NAFLD) was observed in the sixth decade of life. The prevalence of FLD in males increased stably with age, and steadily from 50-60 years of age in females. The peak prevalence was observed in females (31.4%) at the age of 60 years, which was 20 years later than that in males (23.8%), which might be due to the menopausal status and lack of physical exercise in this period of time.

In developed countries, alcoholism, obesity and diabetes are the most common causes for FLD. Alcohol drinking plays a role in FLD[22,23]. In this study, only 0.5% adults in Guangdong Province suffered from ALD, reflecting the low alcohol consumption in South China. For this reason, multivariate analyses failed to demonstrate the role of alcohol drinking in FLD (P = 0.078). NAFLD is a manifestation of metabolic syndrome in liver, including obesity, type 2 diabetes, hypertension and dyslipidemia[15,24-28]. Metabolic syndrome, a common disorder in Western countries, has become a severe problem in China due to alterations in life styles and dietary habits. In this study, the prevalence of FLD increased with BMI and HWR, an index for central obesity. These findings are consistent with the reported data[5,19,29,30]. Our results indicate that dyslipidemia, especially high serum level of TG is a major risk factor for FLD, which also agrees with the results in most studies that the role of TG is more important than TC in the pathogenesis of metabolic syndrome and NAFLD[18,23]. When the serum level of TC in this area is relatively low, it fails to reach the significant level as a risk factor for FLD. The education background is related to the prevalence of FLD, probably because the people with a higher education level have paid more attention to their health and they are more likely to adjust their diet habits and exercise for the prevention of obesity.

There are several methodological limitations in this study. First, although participants were randomly selected, the enrollment of a greater proportion of females and elderly subjects in the study may bring about bias to the results. Second, the large number of immigrant population in Guangdong Province, especially in cities may have also confounded the study. In order to limit these biases, the prevalence rate was standardized for age, gender and residency areas. Multivariate regression analyses were performed to strengthen the reliability of our findings. Third, although histology remains the gold standard for the pathological diagnosis of FLD[31,32], imaging modalities like ultrasonography with a reasonably high sensitivity can identify FLD[32-35]. In this study, the diagnosis of fatty liver was based on ultrasonography. For the ethical reason, it is impossible to perform biopsy in an epidemiological study. In addition, the influence of exercise was not taken into account in this study.

In summary, FLD, especially NAFLD, is common in South China and many risk factors are related to the pathogenesis of this metabolic syndrome.

COMMENTS

Background

Fatty liver disease (FLD) has become a common problem in both developed and developing countries. In recent years, due to alterations in life style and dietary habits, the incidence of FLD has increased dramatically in China. It is thus of importance to assess the epidemiological features of FLD in this country in order to facilitate its prevention and treatment. Although some epidemiological studies concerning FLD have been reported in China, most of them were based on clinical settings or on health check-up groups, which do not represent the population data. So far only one population-based study from Shanghai, an eastern city of China, has been published in English. The data from rural areas and other parts of this country are still lacking.

Research frontiers

FLD encompasses a morphological spectrum consisting of hepatic steatosis and steatohepatitis which can progress to cirrhosis and hepatocellular carcinoma. In the past, excess alcohol consumption accounted for most cases of FLD, but recently nonalcoholic causes for fatty liver have attracted considerable attention. In addition to alcohol consumption, factors such as insulin resistance (IR), oxidative stress, mitochondrial dysfunction, immune deregulation, and adipokines play an important role in the pathogenesis of FLD. Although FLD has been reported worldwide, it is difficult to determine the true prevalence because of problems in interpreting data from various studies due to referral bias, population heterogeneity, study design, imaging modalities, and liver biopsies. These studies on the FLD epidemiology have improved the detailed knowledge about the prevalence of FLD and the metabolic risk factors associated regional (e.g., urban *vs* rural) and ethnic differences, as they may provide clues to pathogenesis and individual risk factors for liver disease and metabolic complications. Further studies are also required to confirm the impact of fatty liver on chronic liver diseases.

Innovations and breakthroughs

The incidence of FLD is likely to rise steadily in China in urban areas owing to the westernization of the diet, excessive food intake, increased elderly population, changes in life style and lack of exercise. Because the majority of people live in rural areas of China, it is necessary to investigate the prevalence of FLD in China. In this study, since the random sampling method and high response rate limited the selection and responder biases, our results reflect the population-based data and the major risk factors for FLD in both urban and rural areas. This study assessed the prevalence of and risk factors for NAFLD in China.

Applications

With regards to increasing obesity, FLD, especially NAFLD, is believed to be the most common form of chronic liver diseases, which can progress to cirrhosis. Because there is no proven management for NAFLD, identification of important risk factors for this condition will provide valuable information on both risk stratification and development of risk-reduction strategies.

Terminology

Fatty liver disease (FLD), defined as lipid accumulation exceeding the normal range of 5% of liver wet weight, is a kind of chronic disorders, including alcoholic liver disease (ALD) and nonalcoholic fatty liver diseases (NAFLD). Briefly, the diagnosis of FLD, NAFLD and ALD is based on the combination of medical history, clinical symptoms, laboratory and imaging modalities (ultrasonography, computed tomography scans, and magnetic resonance imaging). Viral hepatitis and other chronic liver diseases need to be ruled out. ALD is diagnosed when a subject fulfilling the FLD criteria drinks more than 40 g (male) or 20 g (female) alcohol per day over 5 years. NAFLD is diagnosed when alcohol consumption is less than 40 g (male) or 20 g (female).

Peer review

This is an interesting, well written and generally well designed study. Although it does not identify fundamentally new or unexpected findings with respect to the incidence of FLD, the data highlighting the nonalcohol - related incidence of FLD

in developing areas are important.

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RAPID COMMUNICATION

Antitumor and antiangiogenic activities of anti-vascular endothelial growth factor hairpin ribozyme in human hepatocellular carcinoma cell cultures and xenografts

Li-Hua Li, Zi-Jian Guo, Ling-Ling Yan, Ji-Cheng Yang, Yu-Feng Xie, Wei-Hua Sheng, Zhao-Hui Huang, Xue-Hao Wang

Li-Hua Li, Zi-Jian Guo, Ling-Ling Yan, Zhao-Hui Huang, Oncology Institute of Wuxi, the Fourth Affiliated Hospital of Soochow University, 200 Huihe Road, Wuxi 214062, Jiangsu Province, China
Ji-Cheng Yang, Yu-Feng Xie, Wei-Hua Sheng, Department of Cellular and Molecular Biology, Medical School, Soochow University, Soochow 215007, Jiangsu Province, China
Xue-Hao Wang, Department of Liver and Biliary Duct Surgery, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Supported by the Grant from Calling for Tenders by Key Subject of Jiangsu Province, No. WK200221
Correspondence to: Zi-Jian Guo, Oncology Institute of Wuxi, the Fourth Affiliated Hospital of Soochow University, 200 Huihe Road, Wuxi 214062, Jiangsu Province, China. gzjwxsy@sina.com
Telephone: +86-510-88682119 Fax: +86-510-88683507
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Abstract

AIM: To study the effectiveness and mechanisms of anti- human vascular endothelial growth factor (hVEGF) hairpin ribozyme on angiogenesis, oncogenicity and tumor growth in a hepatocarcinoma cell line and a xenografted model.

METHODS: The artificial anti-hVEGF hairpin ribozyme was transfected into hepatocarcinoma cell line SMMC-7721 and, subsequently, polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) were performed to confirm the ribozyme gene integration and transcription. To determine the effects of ribozyme ,VEGF expression was detected by semiquantitative RT-PCR and enzyme liked immunosorbent assay (ELISA). MTT assay was carried out to measure the cell proliferation. Furthermore,the transfected and control cells were inoculated into nude mice respectively, the growth of cells in nude mice and angiogenesis were observed.

RESULTS: VEGF expression was down-regulated sharply by ribozyme in transfected SMMC-7721 cells and xenografted tumor. Compared to the control group, the transfected cells grew slower in cell cultures and xenografts, and the xenograft formation was delayed as well. In addition, the microvessel density of the xenografted tumor was obviously declined in the transfected group. As demonstrated by microscopy,reduction of VEGF production induced by ribozyme resulted in a significantly higher cell differentiation and less proliferation vigor in xenografted tumor.

CONCLUSION: Anti-hVEGF hairpin ribozyme can effectively inhibit VEGF expression and growth of hepatocarcinoma *in vitro* and *in vivo*. VEGF is functionally related to cell proliferation, differentiation and tumorigenesis in hepatocarcinoma.

Key words: Vascular endothelial growth factor; Angiogenesis; Hairpin ribozyme; Hepatocarcinoma; Gene therapy

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INTRODUCTION

Rapidly growing tumors routinely outstrip their supply of oxygen and nutrients, and induction of new blood vessels is critical to sustain neoplastic proliferation[1-4]. A number of growth factors have been identified as potential positive regulators of angiogenesis. Among them, vascular endothelial growth factor (VEGF) appears to have a central role in the angiogenic process. VEGF not only is the target of many proangiogenic factors but also regulates molecules that are implicated in endothelial proliferation. It has been suggested that VEGF may be a proximate angiogenic factor through which others act. In fact, over-expression of VEGF is the characteristic of most malignant tumors including hepatocarcinoma[5-9], which is regulated in response to hypoxia[7,10-12] and highly related to microvessel density of cancer, grade of malignance and metastasis[13-15]. It now appears that VEGF also has autocrine functions acting as a survival factor for tumor cells protecting themselves from stresses such as hypoxia, chemotherapy and radiotherapy[16]. Consequently, anti-

VEGF therapies are being actively investigated as potential anti-cancer treatment modalities, either as alternatives or adjuncts to conventional chemo or radiation therapy. Clinical trials using anti-VEGF mAbs such as bevacizumab have validated the efficacy of this therapeutic approach but have also revealed adverse effects.

Hepatocellular carcinoma (HCC) is one of the most common and aggressive cancers worldwide, being the third cause for cancer-related deaths. As a malignant solid tumor, HCC is characterized by fast infiltrating growth, early metastasis, high-grade malignancy, and poor therapeutic efficacy[17]. It is a highly vascular tumor dependenting on neovascularization and a rapidly developing malignancy[18]. The VEGF level in HCC tissues is significantly higher than that in distal cancerous tissues[6]. The abnormal expression levels of VEGF in sera of HCC patients are directly correlated with the metastasis and recurrence of tumors[5,19,20]. Since HCC is insensitive to conventional chemotherapeutics and its prognosis is poor, it is important to explore an antiangiogenesis method for HCC characterized by a high vascularity. Up to now, many strategies for gene therapy have been developed. Ribozymes are a group of catalytically active nucleic acids capable of site-specific cleavage of target mRNAs, thus decreasing mRNA expression and inhibiting the function of target gene[21]. Both hairpin and hammerhead ribozymes are the most commonly used gene therapy models. In contrast to hammerhead ribozyme, the hairpin ribozyme reaction requires an environment closer to human physiology. The hairpin ribozyme uses a catalytic mechanism that does not require metals for cleavage or ligation of substrate RNA. In this regard, it is presently unique among RNA catalysts. The hairpin ribozyme has been approved for its use in gene therapy.

The role of VEGF in HCC proliferation, differentiation and tumorigenesis is unclear. The present study was to study the effectiveness and mechanisms of hairpin ribozyme targeting human VEGF (hVEGF) mRNA on exon 3 on VEGF expression, angiogenesis, oncogenicity and tumor growth in HCC cell cultures and xenografts.

MATERIALS AND METHODS

Cell line and cell cultures

Human hapatocarcinima cell line SMMC-7721 was maintained in our laboratory. Cells were incubated with RPMI 1640 medium (GIBCO BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated calf serum (Sijiqing, Hangzhou, China), 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate in a humidified atmosphere containing 50 mL/L CO_2 at 37℃.

Synthesis of anti-human VEGF hairpin ribozyme and recombinant plasmids

The secondary structure of ribozyme was obtained by the ribozyme- aided design software, and the primers of hairpin ribozyme were synthesized (Shengong, Shanghai, China) based on the third exon of hVEGF as previously described[22]. The sequences of specific primers of ribozyme used are as follows: forward: 5'-GATCCCTGATAAGAATCCAACCAGAGAAACACACGTTGTGGTATATTACCTGGTAG-3' and reverse: 5'-AATTCTACCAGGTAATATACCACAACGTGTGTTTCTCTGGTTGGATTCTTATCAGG-3'. Ribozyme was synthesized in a 50 μL reaction system, which was mixed with 1 μL (25 μmol/L) primers, 5 μL NaCl buffer and 43 μL ddH_2O. Since the gene of anti- hVEGF hairpin ribozyme only has a 50 bp product, after incubation at 94℃ for 2 min followed by annealing of the two primers at room temperature, ribozyme was acquired.

After plasmid pcDNA3.1+ (Invitrogen, San Diego, CA) was digested with restriction endonucleases BamHI and EcoRI (TakaRa, Japan), the purified products of anti-hVEGF hairpin ribozyme were subcloned into them, thus, the recombinant eukaryotic expression plasmid pcDNA3.1+/ribozyme (abbreviated as pcDNA3.1+/RZ) was constructed.

Preparation and identification of SMMC-7721/RZ transgenic cells

SMMC-7721 cells were seeded in 6-well plates at 2 × 10^5 cells per well and cultured as described above. The recombinant eukaryotic expression plasmid pcDNA3.1+/RZ was transfected into SMMC-7721 cells by LipofetamineTM2000 (Invitrogen, San Diego, CA) following the manufacturer's instructions, and positive clones were selected with G418, then SMMC-7721/RZ transgenic cells were obtained. At the same time, two control groups were established: the SMMC-7721 cell control (abbreviated as SMMC-7721 cell) and the SMMC-7721 cells transfected with blank vector of pcDNA3.1+ control (abbreviated as SMMC-7721/pcDNA3.1+ cells).

Integration and transcription of the objective gene in SMMC-7721/RZ transgenic cells were identified by polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR). Genomic DNA was extracted from cells in 3 groups using genomic DNA (Shengong, Shanghai, China). PCR was performed to detect the integration of ribozyme gene in SMMC-7721/RZ transgenic cells. The ribozyme primers (forward: 5'-GGACTTTCCTACTTGGCAGTACATC-3', reverse: 5'-CCACAACGTGTGTTTCTCTGGTTGG-3') were used in this study. Genomic DNA template was amplified with an initial denaturation at 94℃ for 2 min, then 30 cycles at 94℃ for 50 s, at 58℃ for 50 s and at 72℃ for 45 s, followed by at 72℃ for 10 min. To evaluate transcription of the ribozyme gene in SMMC-7721/RZ transgenic cells, RT-PCR was performed. Total RNA was extracted from cells using UNIQ column total RNA extraction kits (Shengong, Shanghai, China). First strand cDNA was synthesized from 2 μg of total RNA with an oligo (dT)18-primer in 20 μL of first strand reaction mix at 37℃ for 1 h. Thirty cycles of PCR were done. First strand cDNA was employed as template and the sequences of specific primers of ribozyme are as follows: forward: 5'-TAGAGAACCCACTGCTTACTGGCT-3', and reverse: 5'-CCACAACGTGTGTTTCTCTGGTTGG-3'.

RT-PCR and ELISA for VEGF expression

SMMC-7721/RZ cells were seeded in 24-well plates at

1×10^5 cells per well. The SMMC-7721 cell control and the SMMC-7721/pcDNA3.1+ cell control were established. Cells were cultured for 72 h, and then the supernatant was harvested. Total RNA was isolated from cells, and then RT-PCR was performed to detect the transcription level of VEGF mRNA in cells. Data were presented and normalized to β-actin. The sequences of PCR primers of VEGF and β-actin are as follows: VEGF (forward: 5'-TGGTAGAGTTCATGGATGTCTATCA-3', reverse: 5'-GCATGGTGATGTTGGACTCCTCA-3'), β-actin (forward: 5'-TGCGTGACATTAAGGAGAAG -3', reverse: 5'-CTGCATCCTGTCGGCAATG-3'). The expression of VEGF protein in supernatant was detected by ELISA assay following the manufacturer's instructions (Jingmei, Shanghai, China).

Cell proliferation detection by MTT assay

SMMC-7721/RZ cells were seeded in 96-well plates at 1×10^4 cells per well. Three control groups were established as follows: non-cell control group, SMMC-7721 cell control group and SMMC-7721/pcDNA3.1+ cell control group. Cells were cultured for 24, 48, 72, 96 h respectively, and viability was assessed using 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (SIGMA, St. Louis. USA) at a final concentration of 0.5 mg/mL. Cells were incubated for 2 h, the medium was aspirated, and the cells were dissolved in acidic isopropanol (90% isopropanol, 0.5% sodium dodecyl sulfate, 40 mmol/L HCl). Optical density was read on a microplate reader at 570 nm using the isopropanol as blank. The inhibitory rate (IR) was calculated as follows: IR (%) = (1-absorbance of the treated wells)/(absorbance of the control wells) × 100%.

Human hepatocarcinoma xenograft experiments

Female athymic BLAB/c nude mice, 4-5 wk of age, were purchased from Shanghai Experimental Animal Company, Chinese Academy of Sciences (Shanghai, China) and housed in a pathogen-free facility. *In vivo* studies approved by the Fourth Affiliated Hospital of Soochow University Animal Care Committee, were conducted in accordance with the institutional and China guidelines. Subconfluent SMMC-7721/RZ transgenic cells or SMMC-7721/pcDNA3.1+ cells were suspended in PBS and injected subcutaneously into the right flanks (2×10^6/0.1 mL) of 5 nude mice in each treatment group. Tumor size was measured every other day using an external caliper. Tumor volumes (V) were determined following the equation: $V = (L \times W^2) \times 0.5$, where L is the length and W is the width of tumor. The standard for tumor formation was the diameter of tumors > 0.5 cm. When the experiment was terminated at the 5th wk after tumor formation, the mice were sacrificed. The tumors were isolated, photographed, snap frozen in liquid nitrogen and stored at -80℃, or fixed in formalin for subsequent multiple assays.

Histologic and immunohistochemical assay

Tumor tissues were formalin-fixed and paraffin-embedded. After deparaffinized and rehydrated, the slides were stained with hematoxylin and eosin (HE) in succession, and finally mounted and visualized under microscope. The number of karyokinesis and pathologic karyokinesis was calculated in each visual field, and ten visual fields were selected randomly in each section.

Immunostaining was performed on 5-μm thick sections. Serial sections of paraffin-embedded tumor tissue were dewaxed and hydrated. After antigen retrieval, its endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution for 5-10 min. The sections were washed in distilled water, and soaked in pH 7.4 phosphate-buffered saline (PBS) for 5 min. Nonspecific binding was blocked by incubation with 3% normal goat serum for 10 min. The sections were incubated with primary antibody, rabbit anti-human VEGF polyclonal antibody or rat anti-murine cell determinant (CD34) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz), at a dilution of 1:100 for 1-2 h at 37℃. Anti-CD34 was used as a pan-endothelial marker for microvessel density (MVD) analysis. After washed with PBS, the sections were incubated for 30 min at 37℃ with biotinylated secondary antibody and then with horseradish peroxidase (HRP)-conjugated streptavidin for 30 min at 37℃. The color was developed with a Vector DAB substrate kit for 1 min and 30 s and counterstained with hematoxylin. All sections were analyzed by conventional light microscopy and digital photography.

MVD was evaluated according to the CD34 endothelial cell immunostaining. For the microvessel counting, positivelystained CD34 was counted in ten visual fields of each slide. Any endothelial cluster positive for CD34 (brown yellow staining) was considered a single countable microvessel.

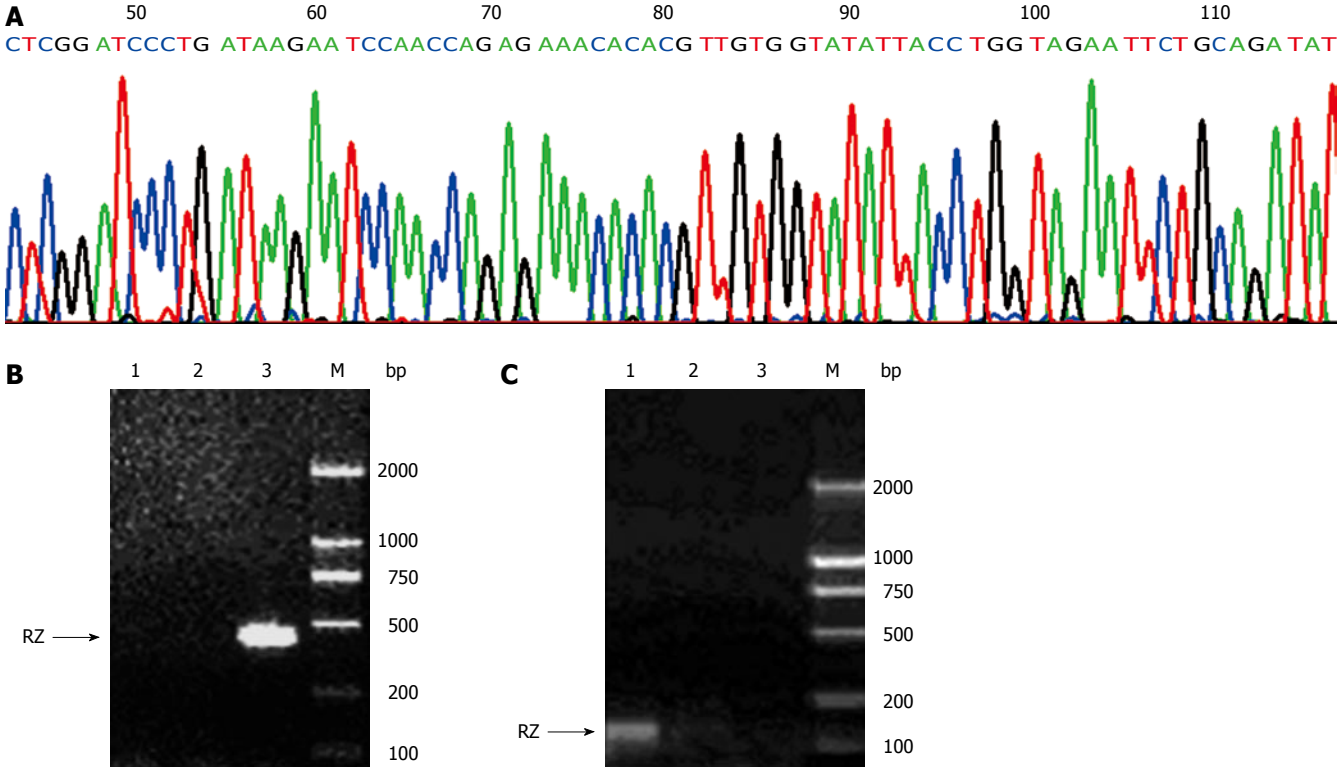
Statistical analysis

All statistical analyses were carried out by the Statistical Package for the Social Sciences (SPSS) software, Version 10.0 for Windows. All values such as tumor volume and weight, karyokinesis and microvessel, were expressed as mean ± SD. Chi-square test was used for rate contrast. $P < 0.05$ was considered statistically significant.

RESULTS

Construction and identification of recombinant eukaryotic expression plasmid pcDNA3.1+/RZ

When products from annealing of the two specific primers of ribozyme were run on agarose gels and visualized with ethidium bromide staining, an amplicon of 50 bp of anti-hVEGF hairpin ribozyme (including cohesive terminal of BamHI and EcoRI) was shown. After cleaved by BamHI and EcoRI, the recombinant eukaryotic expression plasmid pcDNA3.1+/RZ generated an amplicon of 50 bp, revealing that anti-hVEGF hairpin ribozyme was successfully subcloned into pcDNA3.1+. Recombinant plasmids harboring ribozyme sequences in the pcDNA3.1+ backbone were checked by restriction enzyme analysis and sequencing. As shown in Figure 1A, the sequence of the gene subcloned into pcDNA3.1+ was identical to that of the synthetic gene designed according to Genebank, indicating that the recombinant eukaryotic



Figrue 1 Construction and identification of recombinant eukaryotic expression plasmid pcDNA3.1+/RZ. **A**: Sequencing result of the anti-hVEGF hairpin ribozyme gene subcloned into pcDNA3.1+; **B**: Integration of the ribozyme gene detected by PCR (1: SMMC-7721 cells; 2: SMMC-7721/pcDNA3.1+cells; 3: SMMC-7721/RZ cells; M: DL2000 marker); **C**: Transcription of the ribozyme gene detected by RT-PCR (1: SMMC-7721/RZ cells; 2: SMMC-772 cells; 3: SMMC-7721/ pcDNA3.1+ cells; M: DL2000 marker).

expression plasmid pcDNA3.1+/RZ was successfully constructed.

Integration and transcription of anti-hVEGF hairpin ribozyme in SMMC-7721/RZ cells

The forward and reverse primers were designed based on the sequence of pcDNA3.1+ and anti-hVEGF hairpin ribozyme. PCR amplification of ribozyme in genomic DNA of SMMC-7721/RZ cells generated an amplicon of 400 bp (Figure 1B). Using the primers designed based on the sequence of pcDNA3.1+ transcriptional start site and ribozyme, RT-PCR showed that PCR amplification of ribozyme in cDNA of SMMC-7721/RZ cells generated an amplicon of 140 bp (Figure 1C). In contrast, these amplicons were not found in SMMC-7721 cells and SMMC-7721/pcDNA3.1+ cells, demonstrating that the gene of anti-hVEGF hairpin ribozyme transfected into SMMC-7721 cells could be integrated with SMMC-7721 cell genome and transcripted into corresponding mRNA efficiently.

VEGF transcription and expression

As the ultimate goal we sought to achieve was the reduction of VEGF secretion into extracellular compartment by SMMC-7721 cells, we investigated if transfection of ribozyme has a direct effect on VEGF secretion by SMMC-7721 cells. As demonstrated in Figure 2A, VEGF protein expression level detected by ELISA in transgenic SMMC-7721/RZ group was 363.64 ± 19.68 ng/L, which was significantly lower than that in SMMC-7721 cell group (1358.69 ± 49.81 ng/L) and SMMC-7721/pcDNA3.1+ group (1369.57 ± 32.61 ng/L) ($P < 0.01$). There was no significant difference between SMMC-7721 cell group and SMMC-7721/pcDNA3.1+ group ($P > 0.05$). These results indicate that ribozyme inhibited VEGF expression (more than 73%) in HCC cells *in vitro*.

To determine whether the inhibitory effect of ribozyme on VEGF expression is at the transcriptional level, VEGF mRNA expression in tumor cells was detected by RT-PCR. The products were run on 1% agarose gels and visualized with ethidium bromide staining. SMMC-7721/RZ cells and two control group cells generated an amplicon of 195 bp. By normalizing to β-actin, the level of VEGF mRNA expression in SMMC-7721/RZ cells was remarkably lower than that in two control groups (Figure 2B), suggesting that ribozyme could reduce VEGF expression by inhibiting its transcription.

Proliferation of transgenic cells

As shown in Figure 2C-D, after cells were cultured for 24, 48, 72 and 96 h, the proliferation rate for SMMC-7721/RZ cells was significantly lower than that for SMMC-7721 cells and SMMC-7721/pcDNA3.1+ cells ($P < 0.01$). On the third day, ribozyme-mediated growth inhibition rate reached 78.6%. There was no significant difference in proliferation of SMMC-7721 cells and SMMC-7721/pcDNA3.1+ cells ($P > 0.05$). The results reveal that ribozyme inhibited proliferation of HCC cells *in vitro*.

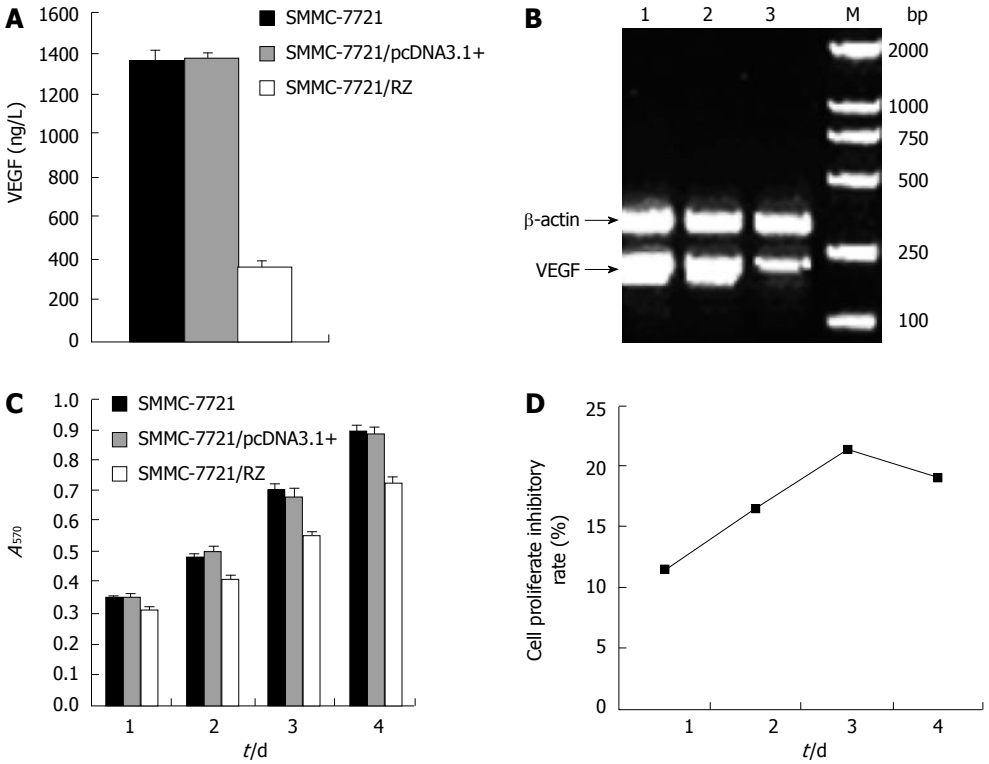


Figure 2 Effects of anti-hVEGF hairpin ribozyme on expression of VEGF and proliferation of SMMC-7721 cells *in vitro*. **A**: ELISA validation of VEGF expression; **B**: RT-PCR validation of VEGF mRNA transcription in SMMC-7721 group(1), SMMC-7721/pcDNA3.1+ group(2) and SMMC-7721/RZ group(3), (M: DL2000 marker); **C**: Influence of ribozyme on SMMC-7721 cell proliferation; **D**: Influence of ribozyme on inhibitory rate for proliferation of SMMC-7721 cells.

Formation and growth of xenografted tumor

To determine the impact of anti-hVEGF hairpin ribozyme on tumorigenicity *in vivo*, control and ribozyme-transduced cells were transplanted subcutaneously into BLAB/c nude mice. Histology and immunohistochemistry displayed different levels of VEGF expression and angiogenesis on sections of xenografted and control tumor tissues. As expected, tumors appeared on d 14 (± 6.3) and d 7 (± 2.6) in SMMC-7721/RZ group and SMMC-7721/pcDNA3.1+ group, respectively ($P < 0.01$). The ribozyme transgenic cells resulted in tumor formation with lower levels of VEGF expression and angiogenesis and delayed tumor formation on 17.2 ± 13.7 d compared with 8.6 ± 3.3 d in control cells ($P < 0.01$). Formation of large, red (vascular) tumors was observed in SMMC-7721/pcDNA3.1+ cells of all mice. However, formation of small white (avascular) tumors was found after inoculation with SMMC-7721/RZ cells. The tumor volume and weight at the time of sacrifice in SMMC-7721/RZ group (0.19 ± 0.0085 cm^3, 0.26 ± 0.076 g) were significantly lower than those in SMMC-7721/pcDNA3.1+ group (0.59 ± 0.019 cm^3, 0.74 ± 0.050 g, $P < 0.01$). The inhibitory effects of anti-hVEGF hairpin ribozyme on HCC xenograft growth in nude mice were comparable and significant ($P < 0.01$).

Histologic and immunohistochemical findings

As shown in Figure 3A, different shape and size of tumor cells were found in SMMC-7721/pcDNA3.1+ group. Their nuclei were large with a disproportional ratio of karyoplasm. Nuclear chromatin was condensed. Several basophilia chromatospherites were seen. Normal karyokinesis (8.26 ± 0.55/visual field) and pathologic karyokinesis (8.52 ± 0.17/visual field) were frequently observed. In contrast, the shape and size of tumor cells in SMMC-7721/RZ group were coincident. Nuclear chromatin was puff. Normal karyokinesis (3.18 ± 0.23/visual field) and pathologic karyokinesis (3.3 ± 0.37/visual field) were significantly less than those in SMMC-7721/pcDNA3.1+ group ($P < 0.01$).

Immunohistochemical staining showed VEGF as light brown particles. VEGF chromatosis was distinctly less or/and weaker in SMMC-7721/RZ group than in SMMC-7721/pcDNA3.1+ group (Figure 3B).

The control mice displayed well vascularized tumors. Tumor blood vessels and their wall were abundant in the control group. In SMMC-7721/RZ group, however, tumor blood vessels were seldom seen with vessel wall frame collapsed. As shown in Figure 3C, a significant difference in the relative intensity of immunostaining for anti-CD34 antibody (an endothelial marker) was observed between the SMMC-7721/RZ group and the control group, indicating that vascularization was decreased in SMMC-7721/RZ group after transfection of anti-hVEGF hairpin ribozyme. MVD in the sections was significantly lower in SMMC-7721/RZ group (3.82 ± 0.88) than in the control group (7.02 ± 0.14, $P < 0.01$).

DISCUSSION

It was reported that ribozyme is used to inhibit cellular targets[23-25]. Among them is the ribozyme-based therapeutics for cancer which might be devised to inhibit tumor growth or to prevent its metastasis. Compared to other gene technologies, the superiorities of ribozyme are as follows: (1) ribozyme has dual effects of cleavage and blockade, (2) it cannot be easily hydrolyzed by nucleic acid enzymes because it can bind to target RNA to

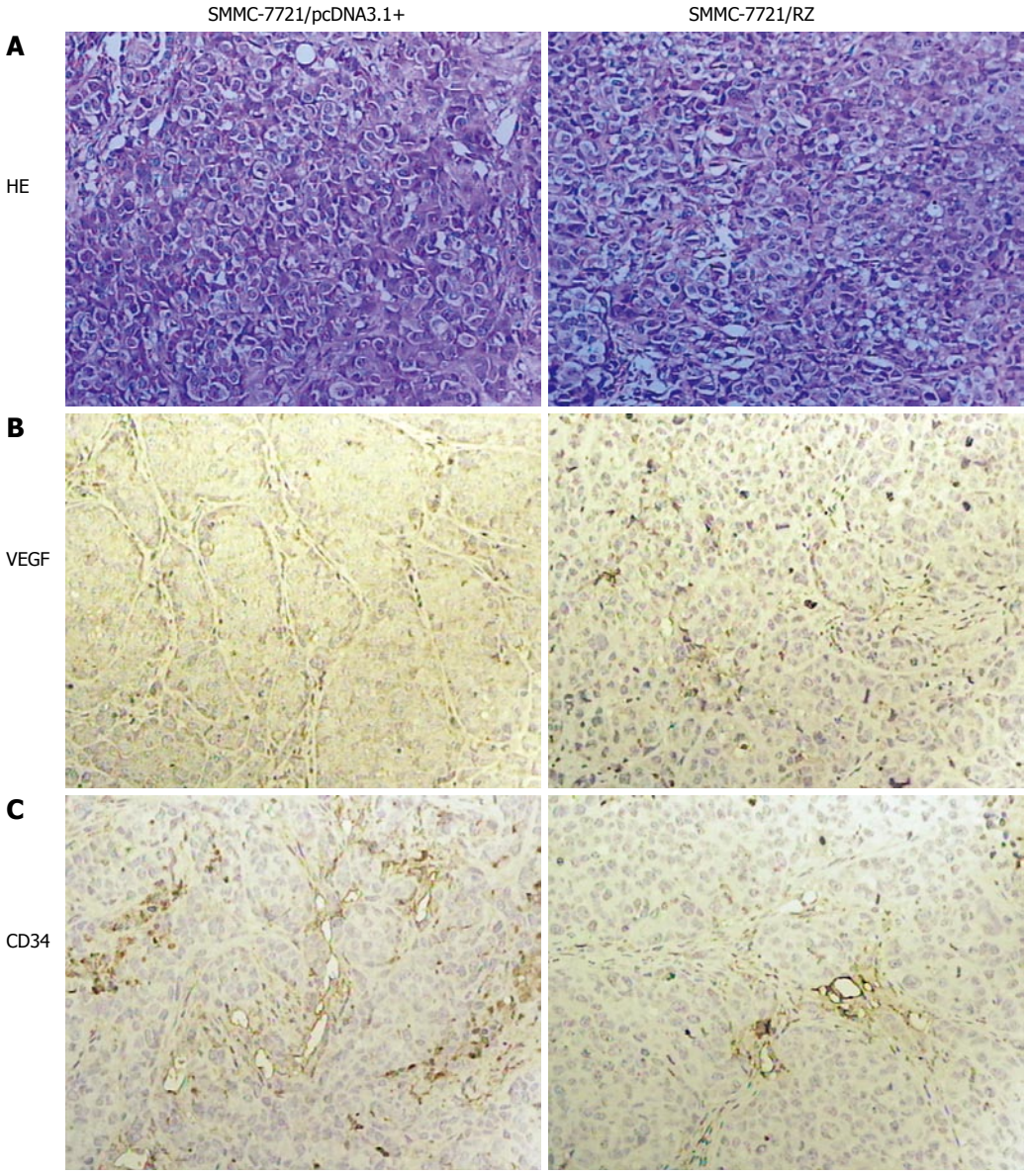


Figure 3 HE staining of tumor tissue (**A**), immunohistochemical staining of VEGF antibody (**B**) and CD34 antibody (**C**) for assay of their positive particles.

form a stable helix structure, (3) ribozyme molecule can destruct multiple target RNAs and be used repeatedly[26]. Biochemical characterization has shown that hairpin ribozyme is one of the most efficient ribozymes.

In order to devise effective anti-hVEGF hairpin ribozymes, we performed a sequence analysis of hVEGF mRNA and studied its secondary structure. We took into account the ideal site of hairpin ribozyme cleavage and its substrates in exon 3 of a GUC since this site is more easy for hairpin ribozyme to approach and less affected by the secondary structure. A fragment of the ribozyme gene and its sequence were verified by digestion of pcDNA3.1+/RZ with BamHI and EcoRI and sequencing. Our results show that the recombinant eukaryotic expression vector pcDNA3.1+/RZ could be successfully constructed. Stable integration and transcription of the ribozyme gene in SMMC-7721 cells were confirmed by PCR and RT-PCR. When introduced into hepatocarcinoma cell line SMMC-7721, anti-hVEGF hairpin ribozyme suppressed not only VEGF mRNA transcription but also VEGF protein expression. VEGF protein expression was inhibited by approximately 73% in SMMC-7721 cells transfected by ribozyme. These results reveal that the constructed ribozyme can selectively inhibit VEGF gene expression in human hepatocarcinoma cells. Ciafre *et al*[27] have developed an anti-VEGF hammerhead ribozyme targeting the 5' part of human VEGF mRNA. Transfection of U87 human glioblastoma cells with plasmid vectors encoding for this ribozyme resulted in a strong (-56%) reduction of VEGF secretion in the extracellular medium. We believe that our ribozyme has a better biological activity in selectively inhibiting VEGF expression. The successful use of ribozymes as therapeutic agent depends upon many factors, e.g., category of ribozymes (hairpin and hammerhead ribozymes), relative amount of active ribozymes in cells, their co-localization with target RNAs, structural features of transcripts influencing accessibility to specific target sites, catalytic efficiency, interaction of target RNAs with proteins, and intracellular stability of targeted RNAs and ribozymes.

Little is known about the role of VEGF in HCC cell proliferation, differentiation and tumorigenesis. One of the more intriguing aspects of our results is that after cells were cultured for 24, 48, 72 and 96 h, the proliferation

rate for SMMC-7721/RZ cells was significantly lower than that for SMMC-7721 cells and blank vector cells, and the inhibitory rate of ribozyme for the growth of SMMC-7721 cells was 21.4% on the third day. Apoptosis peak was detected by flow cytometry in SMMC-7721/RZ cells, the rate of apoptosis was 11.0% (data not shown), suggesting that the expression of anti-hVEGF hairpin ribozyme can significantly reduce VEGF secretion by transfected cells and inhibit cell growth and apoptosis in hepatocarcinoma cells. In other words, VEGF signaling can prolong the survival of HCC cells that may be independent of angiogenesis. Since Masood *et al*[28] reported that the expression of Flt-1 and KDR, the receptors of VEGF, is high in AIDS-Kaposi sarcoma cells and primary tumor tissues. Similar results have been observed in several tumors, by researchers in succession[29-31]. To inhibit VEGF expression or to block VEGF binding to its receptor results in growth inhibition of tumor cell lines, such as melanoma and ovarian carcinoma cell lines expressing VEGF receptors. In sharp contrast, cell lines not expressing VEGFRs show no response[28]. VEGF addition causes phosphorylation of mitogen-activated protein kinase as well as VEGF receptor, and induces proliferation and migration of lung cancer cells[32]. Our results are consistent with those previously observed. These findings suggest that tumor cells secrete VEGF which not only induces angiogenesis but also acts as an autocrine growth factor for carcinoma cells defined by the expression of VEGF receptors[33]. Our data suggest that HCC cells express specific VEGF receptors which respond to autocrine VEGF, thus activating signaling pathways that impede apoptosis and promote cell proliferation.

Because results on cell lines often represent a distorted and incomplete picture of the *in situ* physiopathology of cancer where the tumor microenvironment and neovascularization play a critical role in tumor growth and progression, we expanded our study to matched primary tumors using xenograft models. Oncogenicity is a critical index to judge malignant proliferation. The transfected SMMC-7721 cells were transplanted into nude mice. VEGF expression and microvascularization were distinctly decreased in SMMC-7721/RZ cell group when compared with SMMC-7721/pcDNA3.1+ cell group. Furthermore, tumor formation was delayed and its growth was significantly slowed down by ribozyme. Furthermore, reduced VEGF production induced by ribozyme resulted in higher cell differentiation, less cyto-heteromorphism and proliferation in the ribozyme transfected group, as demonstrated by microscopy, suggesting that VEGF is functionally related to cell differentiation directly or indirectly by regulating expression of certain genes. Our data reveal that VEGF plays a basic role in the induction of tumorigenicity, and underlines the importance of inhibiting its production. Moreover, Xu *et al*[34] found that down-regulation of VEGF gene by introducing anti-VEGF hairpin ribozyme gene into leukemia cell line K562 can alter the gene expression profiles. Among the 4096 gene clones on the microarray, 191 were detected to have the marked changes with 104 down-regulated and 87 up-regulated, showing that they are functionally related to cell cycle progression, gene replication, metabolism, cell apoptosis, cell signal transduction, and oncogenes[34]. All these findings indicate that VEGF is a key molecule for tumor progression, although it is unclear how VEGF expression regulates various genes.

In human hepatocarcinoma tumor xenograft experiment, although there was a significant difference in *P* value between the control and ribozyme groups, tumor formation was observed in the ribozyme group. To further decrease tumor formation, in combined with anti-VEGF ribozyme, it is necessary to explore ribozymes targeting other genes, which contribute to hepatocarcinoma tumorigenesis such as N- ras, C-myc and IGF-Ⅱ.

In conclusion, VEGF promotes tumorigenesis and angiogenesis, and anti-hVEGF hairpin ribozyme can efficiently inhibit the VEGF expression and growth of hepatocarcinoma *in vitro* and *in vivo*. In VEGFRs-expressing tumors such as HCC, VEGF inhibition may be attributed to inhibition of tumor angiogenesis and direct effects on tumor cell proliferation/survival. Anti-hVEGF hairpin ribozyme may be a candidate for HCC gene therapy. However, its clinical application needs further study.

COMMENTS

Background

Angiogenesis, known as the development and proliferation of new blood vessels, is vital for the growth of tumors. Vascular endothelial growth factor (VEGF) is an essential cytokine in the regulation of angiogenesis. Little is known about the role of VEGF in hepatocellular carcinoma (HCC) proliferation, differentiation and tumorigenesis. The present study was to study the effectiveness and mechanisms of anti-human VEGF (hVEGF) hairpin ribozyme on VEGF expression, oncogenicity and tumor growth in hepatocarcinoma cell line and xenografted model.

Research frontiers

HCC is a highly vascular tumor depending on neovascularization and insensitive conventional chemotherapeutics. It is important to explore antiangiogenesis methods for HCC. Hairpin ribozyme has been used in gene therapy.

Innovations and breakthroughs

To our knowledge, this is the first report that anti-hVEGF hairpin ribozyme can effectively inhibit VEGF expression and growth of hepatocarcinoma *in vitro* and *in vivo*. VEGF inhibition can inhibit tumor angiogenesis and tumor growth that may be dependent and independent of angiogenesis in HCC. Our results also suggest that VEGF is functionally related to cell proliferation, differentiation and tumorigenesis.

Applications

Anti-hVEGF hairpin ribozyme can be used as a therapeutic agent for hepatocarcinoma.

Terminology

Ribozymes: a group of catalytically active nucleic acids capable of site-specific cleavage of target mRNAs, thus decreasing mRNA expression and inhibiting the function of target gene.

Peer review

In this paper, the role of anti-VEGF hairpin ribozyme in antitumor and antigangiogenic activities is described. The experiments were well designed. The data support that VEGF plays its role through angiogenesis and other pathways directly leading to carcinoma.

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CASE REPORT

Complete pathological response following down-staging chemoradiation in locally advanced pancreatic cancer: Challenging the boundaries

AZ Khan, V Pitsinis, SS Mudan

AZ Khan, V Pitsinis, SS Mudan, Department of Gastrointestinal Surgery, Royal Marsden Hospital, Fulham Road, London, United Kingdom
Correspondence to: Aamir Z Khan, FRCS Eng (Gen), Department of Gastrointestinal Surgery, Royal Marsden Hospital, Chelsea, London SW3 6JJ, United Kingdom. aamirzkhan@yahoo.com
Telephone: +44-207-3528171 Fax: +44-207-8082988
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Abstract

Pancreatic cancer is an aggressive malignancy, relatively resistant to chemotherapy and radiotherapy, which usually presents late. Disease specific mortality approaches unity despite advances in adjuvant therapy. We present the first reported case of complete pathological response following neoadjuvant therapy in a locally advanced pancreatic adenocarcinoma.

Key words: Chemotherapy; Pancreatic cancer; Pathology

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INTRODUCTION

Pancreatic cancer is an aggressive intra-abdominal malignancy and approximately 3000 new cases are diagnosed each year in the United Kingdom, most of them usually present with advanced pancreatic cancer and only 10%-20% of patients are resectable at presentation. Surgery with complete resection of the primary lesion offers the only chance of long term survivorship and cure in this cohort of patients. For locally advanced pancreatic cancer, several chemotherapeutic and combined modality regimens have been described, generally with conflicting results, poor response rates, high toxicity, not changing the natural history of the disease. Several novel and new regimens are being tried in the neoadjuvant setting in patients with locally advanced pancreatic cancer at presentation in the hope of down-staging the disease and rendering it resectable. We present the first case of complete pathological response following down-staging chemotherapy.

CASE REPORT

A 69-year-old man presented with nausea, anorexia and weight loss. On investigation, he was diagnosed to have locally advanced adenocarcinoma of the pancreas encasing the superior mesenteric artery and vein with enlarged peri-pancreatic and aortocaval nodes (Figure 1). Final staging after endoscopic ultrasound was T4N1M0. The patient had a past medical history of aortic stenosis and atrial fibrillation for which he took diltiazem daily. Shortly after diagnosis he developed obstructive jaundice which was relieved by an endobiliary stent, which failed to achieve adequate drainage, following which he underwent a double bypass in the form of a gastrojejunostomy and hepatojejunostomy.

After a CT guided biopsy which confirmed invasive adenocarcinoma of the pancreas, he commenced chemotherapy with gemcitabine in combination with oxaliplatin. Upon completion of two cycles of chemotherapy, gemcitabine was replaced with capecitabine due to drug interaction causing breathlessness and he underwent 9 cycles of chemotherapy on the new regime. During chemotherapy surveillance cross-sectional imaging suggested stable disease with normal serum CA19-9 levels. After a 4-wk break he further had 6 cycles of the above combination chemotherapy.

Eighteen months from the initial diagnosis surveillance scans demonstrated good local response to the combination chemotherapy without any distant metastatic disease and he was referred for consolidation radiotherapy, of which he had a total of 25 fractions, which he tolerated well and CT scans suggested further reduction in the disease which now looked resectable (Figure 2).

Based on the remarkable response the patient underwent surgery with curative intent in the form of pancreatoduodenectomy approximately 2 years after his initial diagnosis from which he made an uneventful recovery. Histopathology review of the resected specimen showed a complete response with no evidence of the original invasive adenocarcinoma. A follow-up CT scan at 4 mo as shown did not demonstrate any evidence of recurrent disease.

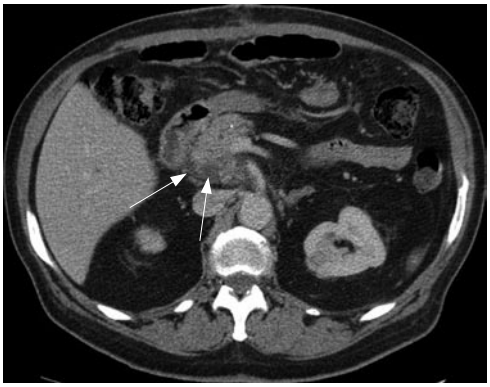

Figure 1 Initial CT scan showing the inoperable mass in the pancreatic head.

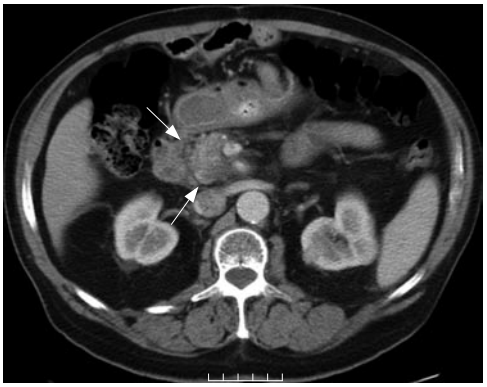

Figure 2 CT scan after completion of chemotherapy showing the operable disease.

DISCUSSION

Pancreatic cancer remains a formidable challenge to clinicians and carries a tumour specific mortality of nearly 100%[1]. Complete resection offers the only hope of cure but is applicable to the minority of patients that present with resectable disease. Approximately 10%-20% of patients present with locally advanced pancreatic cancer without evidence of distant metastatic spread at the time of diagnosis. Prior to the use of neoadjuvant therapy these patients were denied surgery with curative intent and had very short survival times after diagnosis.

Several neoadjuvant regimens have been described to try and downstage locally advanced pancreatic cancer and chemoradiation here is considered as an induction therapy to reduce tumour volume, lymph node disease and extent of vascular involvement[2-5]. In locally advanced pancreatic cancer, complete remissions have not yet been described[6]. Neoadjuvant therapy has its own intrinsic advantages in that it theoretically increases vulnerability of cancer cells because of intact vasculature, better tumour cell oxygenation and probability of sterilizing cells at the resection margin. It also affords a test of the biology of the disease in that patients with progressive disease on chemotherapy can be spared an exploratory laparotomy and trial dissection and managed along the palliative pathway. The risk of pancreatic fistula also seems to be decreased in previously irradiated field[7].

Several regimens have been described and utilized in the neoadjuvant setting for locally advanced pancreatic cancer in an attempt to induce regression of the disease and render it resectable with conflicting results[8]. 5-FU based regimens were originally described but had poor response rates and eventually gave way to gemcitabine based regimens[9]. Combined modality treatments using gemcitabine in combination with other agents like capecitabine, oxaliplatin, bevacizumab and irenotecan have shown promise with prolonged progession-free intervals and better response rates[10-12]. In these settings gemcitobine is used as a radiation sensitizer and combination with external beam radiotherapy remains a popular regimen[13].

Complete pathological response remains an elusive goal in this subset of patients who may then be offered a pancreatoduodenectomy with the aim of long term surviviorship or cure, who if untreated have a very short life expectancy. The future of combined modality therapy for locally advanced pancreatic cancer will focus on the development of newer biological agents that target specific alterations in the cancer cells including vascular endothelial growth factor, epidermal growth factor and matrix metallo protineases[14,15].

In conclusion, complete pathological response following down-staging chemotherapy is possible in patients with locally advanced pancreatic cancer and may offer the patient a chance at long term survival or cure.

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CASE REPORT

Metastatic hepatocellular carcinoma of the external auditory canal

Ryuji Yasumatsu, Kenji Okura, Yumiko Sakiyama, Makoto Nakamuta, Taisei Matsumura, Satoru Uehara, Tomoya Yamamoto, Shizuo Komune

Ryuji Yasumatsu, Department of Otorhinolaryngology, Graduate School of Medical Sciences, Kyushu University and Department of Otorhinolaryngology and Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka 812-8582, Japan
Kenji Okura, Tomoya Yamamoto, Department of Otorhinolaryngology and Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka 812-8582, Japan
Yumiko Sakiyama, Makoto Nakamuta, Department of Gastroenterology and Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka 812-8582, Japan
Taisei Matsumura, Satoru Uehara, Department of Radiology and Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka 812-8582, Japan
Shizuo Komune, Department of Otorhinolaryngology, Graduate School of Medical Sciences, Kyushu University, Japan
Correspondence to: Ryuji Yasumatsu, Department of Otorhinolaryngology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. yasuryuj@qent.med.kyushu-u.ac.jp
Telephone: +81-92-6425668 Fax: +81-92-6425685
Received: August 29, 2007 Revised: September 28, 2007

Abstract

This report describes a rare case of metastatic hepatocellular carcinoma (HCC) presenting a huge mass in the left external auditory canal (EAC). The patient was a 55-year-old man with hepatitis B virus-related HCC. He presented to our department with a three-month history of increasing left otalgia, and hearing loss with recent fresh aural bleeding. Histopathologic examination indicated that the tumor was secondary to HCC. Although external irradiation was not effective, the tumor was treated with surgical debulking and high dose rate 192 Ir remote afterloading system (RALS) for postoperative intracavitary irradiation. A review of the literature revealed only five other cases of HCC metastasis to the temporal bone, all of which mainly metastasized in the internal acoustic meatus. The present case is the first report of HCC metastasis to the EAC.

Key words: Hepatocellular carcinoma; Metastasis; External auditory canal

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a very highly invasive tumor that metastasizes hematogenously and lymphogenously to distant sites. Most frequently affected sites are the lungs, regional lymph nodes, bones, and adrenal glands[1]. However, external auditory canal (EAC) metastases from HCC are extremely rare. A review of the literature revealed only five other cases of HCC metastasis to the temporal bone, all of which mainly metastasized in the internal acoustic meatus[2-4]. To the best of our knowledge, the present patient is the first case of HCC metastasis to the EAC.

We report here a rare case of bleeding due to EAC metastasis from HCC. The patient was treated with surgical debulking and high dose rate ^{192}Ir remote afterloading system (RALS) for postoperative intracavitary irradiation.

CASE REPORT

A 55-year-old man presented to our department with a three-month history of increasing left otalgia, and hearing loss with recent fresh aural bleeding, in April 2006. He denied any vertigo, and had no evidence of facial palsy. His past medical history revealed that hepatitis B virus-related HCC was diagnosed two years ago. Owing to the tumor's inoperability and the presence of lung metastases, the patient began systemic chemotherapy with 5-fluorouracil and cisplatin, which was maintained for two years.

Physical examination did not find the left tympanic membrane, because the EAC was completely occupied by a tumor. Right-sided otoscopy was normal. Vestibular examination was unremarkable. There was no lymphadenopathy, and the remainder of the head and neck and neurologic examinations was unremarkable.

Magnetic resonance imaging showed a well-enhanced tumor lesion within the left EAC. T1- and T2- weighted images revealed that the EAC lesion exhibited a mixture of hypointensity and hyperintensity (Figure 1A). CT imaging showed tumor formation on the posterior wall of the EAC, and a shallow bony erosion (Figure 1B).

A biopsy was performed, and pathologic examination

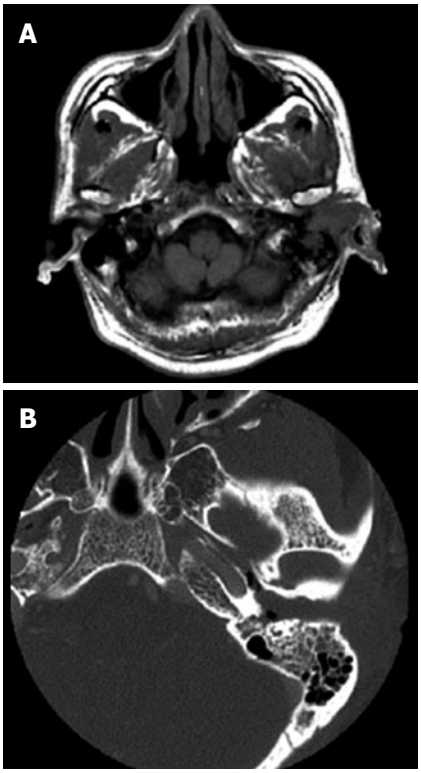

Figure 1 Axial T1-weighted magnetic resonance image of the head showing a huge mass in the left EAC with extension of the lesion into the mastoid (**A**), and axial CT of the temporal bone demonstrating the mass occupying the left EAC (**B**).

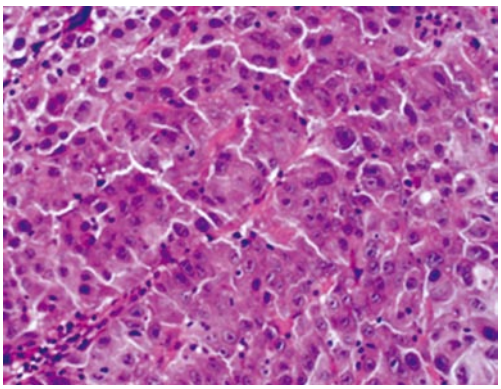

Figure 2 Microscopically, the lesion in the external auditory canal is composed of proliferating atypical epithelial cells forming a solid and thick trabecular arrangement with necrosis, and a fibrous stroma (hematoxylin/eosin stain, x 400).

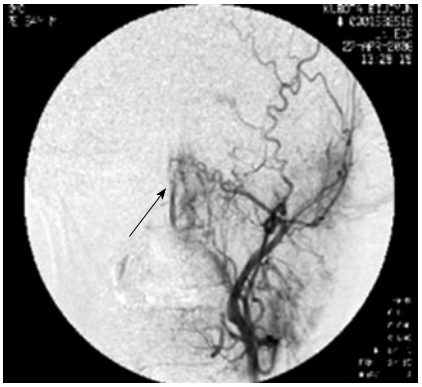

Figure 3 Angiography showing hypervascularity in the left external auditory canal supplied by the posterior auricular artery (the arrow indicates the tumor stain).

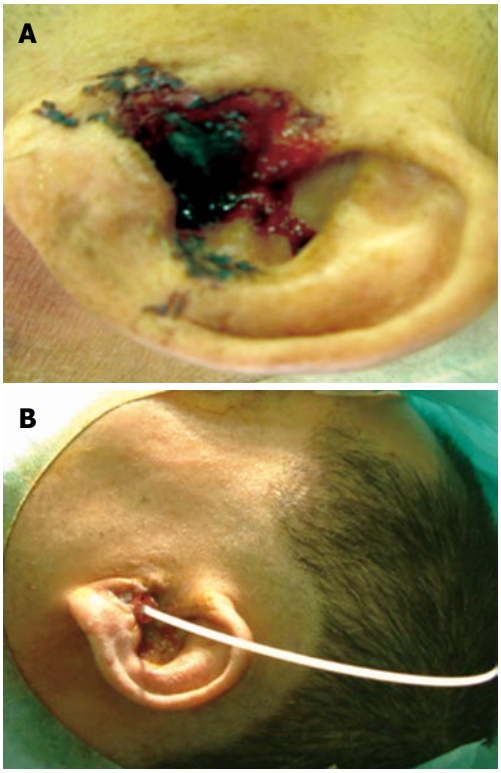

Figure 4 Metastatic hepatocellular carcinoma protruding from the left external auditory canal (**A**) and inserted and fixed catheter for the ^{192}Ir remote afterloader system in the left external auditory canal after debulking surgery (**B**).

taken through the EAC suggested metastasis of HCC with histopathologic features similar to the primary lesion (Figure 2). The biopsy of the mass produced much bleeding, which was stopped with local pressure.

First, the patient received external irradiation (total dose, 30 Gy) to treat the EAC lesion. Despite the external irradiation therapy, the tumor increased in size, and was continuously bleeding. Therefore, it was decided to change the treatment from external irradiation to surgical debulking and ^{192}Ir RALS. We planned to proceed with the debulking surgery for the progressive disease followed by transcatheter arterial embolization by means of superselective catherization.

Angiography was performed to delineate blood supply to the mass, and to embolize any feeding vessels. The external carotid artery was cannulated via the femoral artery, using the Seldinger technique. Evidence of vascular blush in the region of the left EAC was observed, and the posterior auricular artery was identified for embolization (Figure 3).

Following angiography, surgical debulking was performed under general anesthesia and a 6-Fr catheter for the ^{192}Ir remote afterloader system was inserted into the left EAC (Figure 4A and B). A total dose of 15 Gy (10 Gy/Fr/wk) was given over a one-week period. When the irradiation treatment was completed in May 2006, otoscopy revealed tumor remission. Neither radiation-induced dermatitis nor otitis media with effusion was observed. Although the patient remained clinically free of disease in his left EAC (Figure 5), he died of progressive liver failure and lung metastases from HCC 9 mo after the treatment.

DISCUSSION

HCC is the most common malignant tumor of the

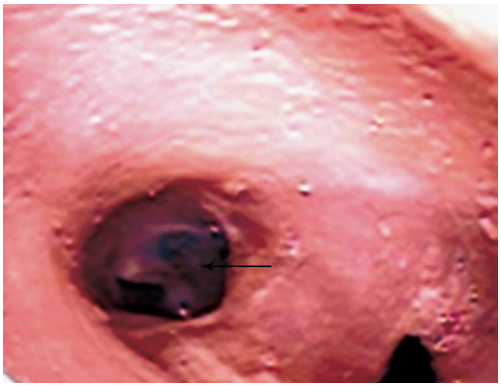

Figure 5 Fiberscopy of the external auditory canal 6 mo after treatment showing no evidence of residual tumor (the arrow indicates the left tympanic membrane).

liver. Intrahepatic metastases occur early in the disease, and more than half of these tumors metastasize to extrahepatic sites[5]. Autopsy and surgical series have suggested the presence of metastases from HCC in the lungs (18.1%-49.2%), lymph nodes (26.5%-41.7%), bones (4.2%-16.3%), and adrenal glands (8.4%-15.4%)[6-8]. However, reports on metastases from distant primary malignancies to different sites in the ear including EAC, are uncommon. The temporal bones are among the more common sites of the ear to develop metastases from distant malignancies[9]. Gloria-Cruz *et al*[4] reviewed the autopsy records of 864 cases, and reported that 47 of 212 patients (22%) with primary non-disseminated neoplasms had metastatic diseases involving the temporal bones. The primary site of the majority of metastases to the temporal bones is the breast (21%), followed by the lungs (12%), kidneys, and prostate, and only three patients (6%) showed histologic features consistent with HCC[4]. No other study has reported HCC metastasis to the EAC. To the best of our knowledge, the present patient is the first case of HCC metastasis to the EAC.

To treat metastatic tumors including HCC of the EAC, curative surgical resection is sometimes impossible, radiation therapy and chemotherapy can be considered as palliative therapies. Although external irradiation was performed in the present case, it was not effective, and the tumor was continuously bleeding. Therefore, we attempted surgical debulking and ^{192}Ir RALS as postoperative irradiation. As a result, fiberscopy after treatment confirmed the effectiveness and safety of this combined procedure. In general, side effects of conventional external irradiation to the auditory canal include many complications such as external canal stenosis, otitis media with effusion, cholesteatoma, sensorineural hearing loss, vestibular impairment, facial nerve paralysis, and osteoradionecrosis. However, none of these complications was observed in this case.

^{192}Ir RALS is the best choice of treatment to ensure decreased doses to other important organs such as the tympanic membrane, temporal bone, and carotid artery while providing a curable dose to a target volume in EAC tumors[10]. In addition, this treatment can be performed in a short period, without any invasive procedure, and may become a choice of modality for its efficacy and less severe side effects.

In conclusion, a rare patient with EAC metastasis from HCC can be treated with surgical debulking and high dose rate ^{192}Ir RALS for postoperative intracavitary irradiation. This combined treatment can be used as a palliative modality for such patients.

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CASE REPORT

Decompensated porto-pulmonary hypertension in a cirrhotic patient with thrombosis of portocaval shunt

Chiara Giannarelli, Antonio De Giorgi, Ferdinando De Negri, Franco Carmassi

Chiara Giannarelli, Antonio De Giorgi, Ferdinando De Negri, Franco Carmassi, Department of Internal Medicine, University of Pisa, via Roma 67, Pisa 56126, Italy
Correspondence to: Chiara Giannarelli, Department of Internal Medicine, University of Pisa, via Roma 67, Pisa 56126, Italy. c.giannarelli@virgilio.it
Telephone: +39-50-992446 Fax: +39-50-553414
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Abstract

We report a case of decompensated porto-pulmonary hypertension closely associated with the development of intra-portocaval shunt thrombosis. A woman with Laennec's cirrhosis was hospitalized because of severe dyspnea and edema. She underwent surgical portocaval anastomosis ten years ago. Imaging studies showed massive intra-shunt thrombosis, portal hypertension, ascites, pleuro-pericardial effusions and enlargement of right cardiac cavities. Cardiac catheterization allowed to rule out coronary and left-sided heart abnormalities and led to the diagnosis of pre-capillary pulmonary hypertension. Antithrombotic treatment with low molecular weight heparin was instituted. The management also included ACE inhibitors, spironolactone, low-salt diet and lactulose. The patient was discharged and three months later we observed the disappearance of edema, ascites and pleuro-pericardial effusions, a marked body weight reduction and improved dyspnea and liver function tests. A possible link between the development of intra-shunt thrombosis and clinical decompensation in our patient was hypothesized. In fact, it has been demonstrated that the increased portal pressure, caused by occlusion of portosystemic shunt, reduces renal plasma flow and increases systemic endothelin-1 concentration. In our patient the disappearance of edematous state and improved dyspnea observed after recanalization of the shunt strongly support this hypothesis.

Key words: Porto-pulmonary hypertension; Porto-caval shunt; Thrombosis

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INTRODUCTION

Porto-pulmonary hypertension (PPHT) refers to the development of pulmonary arterial hypertension in the setting of portal hypertension with or without chronic liver disease and is defined by a mean pulmonary artery pressure > 25 mmHg in the presence of normal pulmo-capillary wedge pressure (< 15 mmHg)[1,2]. The pathogenesis of PPHT is under investigation, although histopathologic features are similar to those found in primary pulmonary hypertension[3]. A current study supports the hypothesis that pulmonary vasculature may be exposed to either cytokines or excess circulating vasoconstrictors, such as endothelin-1 (ET-1) produced by the diseased liver[4]. Although the vast majority of patients with PPHT are asymptomatic, dyspnea is the most frequent presenting symptom[5].

CASE REPORT

A 39-year-old obese woman with Laennec's cirrhosis (Child-Pugh B) was admitted to our hospital in January 2003 because of the recent onset of dyspnea (NYHA IV), dependent edema and abdominal pain. In 1991, she underwent surgical side to side portocaval shunt for refractory ascites and since then she has never complained of dyspnea or edema. Physical examination showed platypnea, arterial blood gas analysis showed hypoxaemia (pO2: 63 mmHg) with orthodeoxia (pO2: 49 mmHg). On admission, liver function tests were as follows: total bilirubin: 84.5 μmol/L, AST: 1.14 μkat/L, ALT: 0.55 μkat/L, GGT: 87 U/L, alkaline phosphatase: 4.2 nkat/L, serum albumin: 28 g/L, ammonia (as NH3): 88 μmol/L, prothrombin time 60%. Hepatic ultrasonography with Doppler imaging showed massive intra-shunt thrombosis, portal hypertension (18 mmHg) and mild ascites. No gastroesophageal varices were found by digestive endoscopy. D-dimer level was 1.4 mg/L and antithrombin activity was 43%. The presence of both peripheral venous thrombosis and recurrent microembolism was ruled out by Doppler ultrasonography and ventilation-perfusion lung scan. Computerized tomography confirmed recent intra-shunt thrombosis and showed also pleuro-pericardial effusions and ascites. Markers of autoimmunity were negative.

Right atrial and ventricular enlargement with severe

Table 1 Hemodinamic parameters during cardiac catheterization

Aortic pressure (mmHg)	103-76 (mean 85)
Right atrial pressure (mmHg)	11 (mean)
Right ventricular pressure (mmHg)	70-18 (mean 35)
Pulmonary arterial pressure (mmHg)	70-30 (mean 43)
Pulmonary capillary wedge pressure (mmHg)	10
Cardiac output (l/min)	5.43
Cardiac index (l/min per m^2)	2.83
Total pulmonary resistance (dyn•s/cm^5)	632
Pulmonary vascular resistance (dyn•s/cm^5)	488
Systemic vascular resistance (dyn•s/cm^5)	1088

tricuspidal regurgitation was also detected by echocardiography. Systolic pulmonary arterial pressure, as measured by Doppler analysis, was 50 mmHg. Cardiac catheterization showed no coronary and left-sided heart abnormalities. Hemodynamic parameters (Table 1) led to the diagnosis of moderate precapillary pulmonary hypertension. Antithrombotic treatment with low molecular weight heparin was instituted. The treatment also included ACE inhibitors, spironolactone, insulin, low-salt diet (2 gr pd) and lactulose. This management decreased the retention of sodium and water in the kidney from 12 mEq/d on admission to 186 mEq/d after 3 d of treatment, and improved edema, as demonstrated by natriuresis. The patient was discharged one month later with the above-mentioned prescription and, after three months of follow-up, physical examination showed the disappearance of edema, marked weight reduction (20 kg) and improved dyspnea (NYHA II). Liver function tests were as follows: total bilirubin: 15.3 μmol/L, AST: 0.56 μkat/L, ALT: 0.47 μkat/L, GGT: 36 U/L, alkaline phosphatase: 1.85 nkat/L, serum albumin: 30 g/L, ammonia (as NH3): 76 μmol/L, prothrombin time 67%. Abdominal ultrasonography showed recanalization of porto-caval shunt, disappearance of ascites and right pleural effusions. No pericardial effusions were found by ecocardiography. Antithrombin activity was 56%, D-dimer 0.2 mg/L. Orthotopic liver transplantation was excluded because of the high intra-operative mortality in patients with portopulmonary hypertension and the conflicting results reported in this condition[6]. Therefore, the patient was referred to a specialized center in order to start an appropriate vasodilatory therapy, which appears to be the only feasible treatment.

DISCUSSION

This case report suggests a possible link between the development of intra-shunt thrombosis and clinical decompensation in our patient. In fact, a recent study showed that increased portal pressure caused by occlusion of porto-systemic shunt reduces renal plasma flow, leading to renal sodium and water retention, and increases systemic ET-1 concentration[4]. In our patient, disappearance of edema and improved dyspnea, observed after recanalization of the shunt, strongly support this hypothesis, although further experience with similar cases is needed.

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CASE REPORT

Malignant fibrous histiocytoma presenting as hemoperitoneum mimicking hepatocellular carcinoma rupture

Hsin-Chi Chen, Chi-Jen Chen, Chin-Ming Jeng, Chan-Ming Yang

Hsin-Chi Chen, Chan-Ming Yang, Department of Radiology, Cathay General Hospital, Hospital Sijhih Branch, No. 2, Lane 59, Jiancheng Road, Sijhih City, Taipei, Taiwan, China
Chi-Jen Chen, Department of Radiology, E-Da Hospital, No. 1, E-Da Road, Jiau-Shu Tsuen, Yan-Chau Hsiang, Kaohsiung, Taiwan, China
Chin-Ming Jeng, Department of Radiology, Cathay General Hospital, No. 280, Jen-Ai Road, Section 4, Taipei, Taiwan, China
Correspondence to: Hsin-Chi Chen, Department of Radiology, Cathay General Hospital, Hospital Sijhih Branch, No. 2, Lane 59, Jiancheng Road, Sijhih City, Taipei, Taiwan, China. hsinchi.chen715@msa.hinet.net
Telephone: +886-2-86463655 Fax: +886-2-87921723
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Abstract

Malignant fibrous histiocytoma (MFH) is a pleomorphic mesenchynal sarcoma. It is uncommonly arises primarily from the intra-peritoneal cavity. Primary peritoneal MFH with tumor bleeding and rupture is rare. We describe the imaging features of a 70-year-old patient presenting with ruptured hemorrhagic peritoneal MFH at subhepatic area, accompanied by massive hemoperitoneum, mimicking a ruptured pedunculated hepatocellular carcinoma. Computed tomography (CT) revealed a large heterogeneous enhanced subhepatic mass with adjacent liver, gallbladder and colon invasion. Tumor hemorrhage and rupture complicated with peritoneal seeding and massive bloody ascites were also detected. Angiography showed a hypervascular tumor fed by enlarged right hepatic arteries, cystic artery and omental branches of gastroepiploic artery. The patient underwent laparotomy for tumor resection, but the tumor recurred one month after operation. To our knowledge, the CT appearance of ruptured intraperitoneal MFH complicated by hemoperitoneum has not been previously described.

Key words: Malignant fibrous histiocytoma; Peritoneum; Hemoperitoneum; Spontaneous rupture; Computed tomography

Chen HC, Chen CJ, Jeng CM, Yang CM. Malignant fibrous histiocytoma presenting as hemoperitoneum mimicking hepatocellular carcinoma rupture. *World J Gastroenterol* 2007; 13(47): 6441-6443

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INTRODUCTION

Massive hemoperitoneum is an emergent life-threatening condition requiring prompt management. Tumor-associated hemoperitoneum is commonly related to hypervascular intra-abdominal visceral organ tumor rupture, but rarely related to hemorrhage of the hypervascular tumor of primary peritoneal origin. Malignant fibrous histiocytoma (MFH) is the most common sarcoma at late adult life. About 5%-10% of MFH lesions arise from the peritoneal cavity[1], and may develop various degrees of tumor bleeding[1-3]. Herein, we present a case of spontaneously bleeding peritoneal MFH located in the subhepatic area with direct liver invasion presenting as massive hemoperitoneum, mimicking a ruptured pedunculated hepatocellular carcinoma.

CASE REPORT

A 70-year-old man was admitted to the emergency department because of intensifying abdominal pain of a 10-days' duration with aggravation in the recent two days before admission. The patient also complained of body weight loss. Physical examination revealed a pallid face, orthopnea and a distended abdomen with a palpable tender mass in the right upper to middle quadrant of the abdomen. Pulse rate and blood pressure were normal. Mild anemia (hemoglobin of 130 g/L), leukocytosis (white blood cell count 16.83 × 10^9/L) and elevated C-reactive protein (247 mg/L) were also noted. Other laboratory data were unremarkable. Serological tests were negative for hepatitis B and positive for hepatitis C. The serum levels of tumor markers of alpha-fetoprotein, CEA and CA19-9 were within normal limits.

Abdominal sonography revealed a large mixed hyperechoic and hypoechoic mass at the hepatic right lower region with massive ascites. Abdominal paracentesis revealed bloody ascites, with an erythrocyte count of 100 000/mm^3. Computed tomography (CT) depicted a large ill-defined hemorrhagic mass (approximately 7.5 cm × 8.6 cm × 12.4 cm in size) with heterogeneous enhancement involving segments V and VI of liver with large extra-hepatic components in the peritoneal cavity accompanied with massive bloody ascites (Figure 1). The subhepatic mass also compressed and invaded the gallbladder and adjacent hepatic flexure of colon. Enlarged omental arteries were found surrounding the subhepatic mass (Figure 1). Tumor rupture with irregular tumor margin and peritoneal seeding were also present.

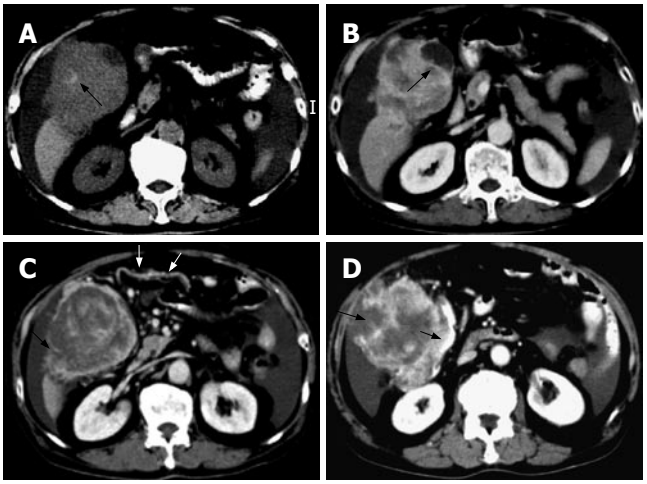

Figure 1 A 70-year-old man with hemorrhagic peritoneal malignant fibrous histiocytoma located subhepatically and presenting as massive hemoperitoneum. **A**: Pre-enhanced computed tomography shows a high-density intra-lesional hemorrhage (black arrow) within the subhepatic tumor involving the anterior aspect of segment V and VI of liver. Ascites are also noted; **B**: Post-enhanced CT shows heterogeneous enhancement of the subhepatic mass directly invading the gallbladder (black arrow); **C**: Post-enhanced CT study at a lower level shows enlarged omental branches (white arrows) of the gastroepiploic artery supplying the subhepatic tumor. A cleavage plane (black arrow) between the subhepatic mass and the liver tip parenchyma was identified, suggestive of an extra-hepatic origin of the mass; **D**: Post-enhanced CT study at the lower tumor level reveals the large subhepatic mass directly invades the adjacent hepatic flexure of colon (thick black arrow). Tumor rupture with irregular tumor margin (thin black arrow) is also evident.

A presumptive diagnosis of pedunculated hepatocellular carcinoma rupture was made. Therefore, emergent angiography was performed and disclosed a large hypervascular tumor located at the right subhepatic region, supplied by enlarged right inferior hepatic arteries, cystic artery, and omental branches of gastroepiploic artery (Figure 2). Transarterial embolization with gelfoam pieces was performed to achieve hemostasis for these engorged feeding arteries.

Six days later, the patient underwent laparotomy for radical tumor excision. Intraoperative examination revealed a large bleeding peritoneal subhepatic tumor rupture with contiguous omentum, liver, gallbladder and colon invasion. Peritoneal seeding and 3 L bloody ascites were also found. Pathology of the excised specimen revealed a storiform-pleomorphic malignant fibrous histiocytoma with marked hemorrhage and necrosis.

One month after the operation, the patient was readmitted to the emergency department due to massive upper gastrointestinal bleeding. CT revealed multiple hepatic and peritoneal metastases with duodenal involvement. Panendoscopy confirmed the duodenal invasion by the recurrent tumor causing internal bleeding. Despite intensive fluid supplementation and blood transfusion therapy, the patient died of hypovolemic shock 3 d later.

DISCUSSION

MFH is a pleomorphic sarcoma initially described by O'Brien and Stout[4]. It is the most common sarcoma in late adulthood, with a peak incidence in the fifth and sixth decades. Men are affected twice as frequently as women. MFH is microscopically characterized by areas of spindle cells arranged in a storiform pattern, and pleomorphic areas with haphazardly arranged sheets of fibroblasts and histiocytes. This neoplasm has a variety of histologic subtypes, including storiform-pleomorphic, myxoid, giant cell, inflammatory and angiomatoid. MFH generally affects the extremities and retroperitoneum. Uncommon locations include the head and neck region, dura mater, brain, lung, heart, aorta, pancreas, liver, spleen, breast, intestine and mesentery[1,5,6]. The propensity of MFH for invasion of contiguous organs and local recurrence result in its poor prognosis.

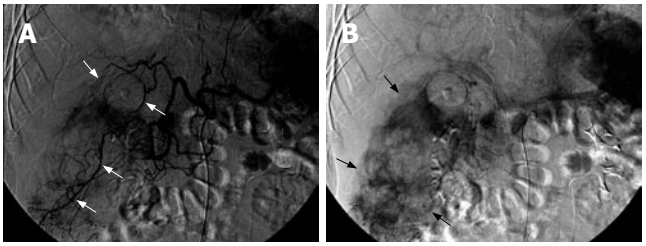

Figure 2 **A**: Arterial phase image of the celiac arteriogram shows hypertrophied right inferior hepatic arteries, cystic artery and omental branches of gastroepiploic artery (white arrows) supplying the hypervascular subhepatic mass; **B**: Venous phase shows prominent tumor stains in the peripheral portion of the huge subhepatic mass (black arrows).

The radiographic features of MFH are non-specific. On CT, FMH usually presents as a poorly demarcated or a well circumscribed mass, and exhibits peripheral solid enhanced component with intralesional hypodense areas of myxoid change, hemorrhage or necrosis. Although the vascularity of MFH is variable, the majority of lesions are moderately hypervascular with tumor supply derived from multiple surrounding vessels[1,7]. Because of the rich vascularity of the lesion, preoperative transarterial embolization may be considered to shrink the tumor and minimize intraoperative hemorrhage.

MFH can develop variable degrees of tumor hemorrhage either spontaneously or as a result of response to chemotherapy[1,8]. Approximately 5% MFHs will develop apparent intra-tumoral hemorrhage spontaneously, especially when MFH arises from the peritoneum or omentum[1]. The intratumoral hemorrhage can be so extensive as to obscure the underlying neoplasms and create a large cyst-like space that might be mistaken as a hematoma, abscess or cystic tumor. Previous reports described that intra-peritoneal MFHs situated at the mesovarium and gastrocolic ligament with marked intra-tumoral hemorrhage manifested as large cystic tumors, causing diagnostic confusion[2-3]. Nevertheless, these intra-peritoneal MFH lesions with extensive tumor hemorrhage were still confined to the tumor mass itself, rather than complicated with extra-tumoral bleeding into the peritoneal cavity. However, in the present case, the subhepatic peritoneal MFH not only experienced intra-tumoral hemorrhage but also developed tumor rupture with extra-tumoral bleeding into the peritoneal cavity, mimicking a ruptured hepatocellular carcinoma. Three previous cases of MFH causing extra-tumoral massive bleeding had

been reported, all involving metastatic MFH lesions at the alimentary tract causing gastrointestinal bleeding[9-11]. A case of post-irradiation induced dermal MFH with spontaneous tumor hemorrhage, causing massive skin bleeding had also been reported[12]. Another case of primary aortic MFH presenting as aortic dissection was described as well[13]. However, to the best of our knowledge, intra-peritoneal MFH bleeding complicated with massive hemoperitoneum causing acute abdominal emergency has not been previously reported.

Tumor-associated hemoperitoneum is frequently related to rupture of underlying intra-abdominal hypervascular tumors. Hepatocellular carcinoma (HCC) rupture is the most common cause of tumor-associated hemoperitoneum in male patients of all ages in Asia and Africa, especially patients with large and peripherally located tumors. Compared to the incidence of intraperitoneal visceral organ tumor rupture causing hemoperitoneum, the occurrence of primary peritoneum-origin tumor bleeding presenting with hemoperitoneum is rare. A review of the literature found only one similar case report which described a hypervascular peritoneal hemangiopericytoma with spontaneous tumor bleeding causing a small amount of hemoperitoneum[14]. By contrast, in our present case, large amounts of bloody ascites were found, indicating that the MFH had tumor rupture with active tumor bleeding rather than minimal tumor bleeding only.

In the present case, CT showed that the vast majority of subhepatic tumor was situated in the extra-hepatic peritoneal cavity and the center of the mass was outside of the liver parenchyma. These findings suggest that the subhepatic mass may be a tumor of extra-hepatic origin rather than arising from the liver parenchyma. Furthermore, the subhepatic tumor was large enough not only to compress but also to directly invade the liver and gallbladder. However, the subhepatic tumor and the compressed liver tip parenchyma still exhibited negative "beak sign" on CT study, seen as an obtuse angle between the subhepatic mass and the compressed liver tip surface with partially preserved dull edges of the liver. These findings suggest that the subhepatic mass did not arise from the liver. Moreover, a cleavage plane between the subhepatic mass and the compressed lower-most liver tip parenchyma was also identified as negative "embedded organ sign" on CT scan, indicating the subhepatic mass was compressing the liver, but did not arise from the liver. In addition, CT also revealed enlarged omental arteries supplying the subhepatic mass, presenting as "prominent feeding artery sign". This suggests the subhepatic mass may be a tumor of peritoneal omentum origin. Angiography also confirmed the subhepatic tumor was mainly supplied by multiple enlarged omental arteries, whereas the hepatic artery only accounted for a minority of tumor feeding. Advanced HCC with omentum invasion can develop extra-hepatic collateral vessels from the omental branches of the gastroepiploic artery, but HCC usually still preserves its dominant feeding arteries from the hepatic artery rather than from the extra-hepatic omental artery. The more prominent blood supply from the omental arteries than the hepatic artery in the present case is an unusual phenomenon in HCC. Careful analysis of the CT imaging features of this case indicate that a subhepatically located bleeding peritoneal tumor with liver invasion should be taken into account as a possible cause of peri-hepatic tumor complicated with hemopertitoneum. Since MFH is the most common sarcoma in the elderly, primary peritoneal MFH, although rare, should be included among the causes of tumor-related hemoperitoneum. However, the radiographic features of MFH are indistinguishable from those of other peritoneal malignant neoplasms, such as leiomyosarcoma, gastrointestinal stromal tumor, fibrosarcoma or angiosarcoma.

In conclusion, MFH arising from the peritoneal subhepatic region is rare, but the potential exists for a hemorrhagic peritoneal sarcoma to directly invade adjacent organs, mimicking an exophytic hepatic tumor rupture. The possibility of MFH should be carefully considered when a patient presents with a hemorrhagic subhepatic tumor and no prior documented hepatitis episode and liver cirrhosis.

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CASE REPORT

Eosinophilic enteritis presenting as a rare cause for ileo-ileal intussusception

Ashok Yadavrao Kshirsagar, Sunil Vitthalrao Jagtap, Rajkamal Prabhudayal Kanojiya, Yogesh Bhupal Langade, Sanjay Laxman Shinde, Niraj Shekhar

Ashok Yadavrao Kshirsagar, Sunil Vitthalrao Jagtap, Rajkamal Prabhudayal Kanojiya, Yogesh Bhupal Langade, Sanjay Laxman Shinde, Niraj Shekhar, Department of Surgery, Department of Pathology, Krishna Institute of Medical Sciences & Deemed University, Dhebewadi Road, Malkapur, Karad 415110, Maharashtra, India
Correspondence to: Dr. Ashok Yadavrao Kshirsagar, Department of Surgery, Krishna Institute of Medical Sciences & Deemed University, Dhebewadi Road, Malkapur, Karad 415110, Maharashtra, India. drayk@indiatimes.com
Telephone: +91-9422400435 Fax: +91-21-64242170
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Abstract

Eosinophilic enteritis, a relatively rare entity, usually involves gastric antrum or proximal small bowel. Our case is rarer in its involvement of the distal small bowel and presents unusually as intussusception. The disease if diagnosed in the initial stages responds well to medical treatment but if associated with complications or misdiagnosed, surgical modality is the treatment of choice. In our case, the patient presented with acute intestinal obstruction due to intussusception and emergency laparotomy with ileoileal anastomosis was done. Histopathology confirmed the diagnosis as eosinophilic enteritis. This case with such a presentation is discussed here.

Key words: Eosinophilic enteritis; Intussusception; Intestinal obstruction; Eosinophils; Ileoileal

Kshirsagar AY, Jagtap SV, Kanojiya RP, Langade YB, Shinde SL, Shekhar N. Eosinophilic enteritis presenting as a rare cause for ileo-ileal intussusception. *World J Gastroenterol* 2007; 13(47): 6444-6445

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INTRODUCTION

Eosinophilic enteritis is a rare, poorly understood condition presenting with a bizarre spectrum of unexplained symptoms mimicking any other acute abdominal conditions. Diagnosis initially is based on exclusion of other abdominal conditions though ultimately histopathology is definitive. The majority of patients respond to medical treatment but if associated with complications like obstruction, perforation, intussusception *etc.*, surgical intervention is necessary. We present a case of eosinophilic enteritis of the distal ileum with intussusception.

CASE REPORT

A 40-year-old man was admitted to the emergency department of our hospital for severe colicky upper abdominal pain and vomiting. He had a history of mild colicky abdominal pain a month ago but no history of food sensitization, allergic disease, asthma, parasitic infestations or any abdominal surgery. Physical examination revealed generalized distention with tenderness all over the abdomen. Per rectal examination was unremarkable. Blood investigations revealed 117 g/L hemoglobin, 8700/mm^3 white blood cells with 67% neutrophils, 36% lymphocytes and 3% eosinophils. The rest of haematological investigations were within normal limits. Abdominal radiograph revealed multiple air-fluid levels suggestive of small bowel obstruction. Ultrasonography revealed intussusception of the distal small bowel. Emergency laparotomy revealed ileo-ileal intussusception with gangrenous changes, about 15 cm proximal to the ileo-caecal junction. Resection with ileo-ileal anastomosis was done.

Histopathology revealed the surrounding mucosa and submucosa. Muscular layers were heavily infiltrated by eosinophils (50-60/Hpf) along with polymorphonuclear cells, lymphocytes and few plasma cells. Intestinal wall was thickened with areas of necrosis suggestive of eosinophilic enteritis with gangrene. No evidence of malignancy was found. The patient was asymptomatic, his follow-up serum immunoglobulin E levels were within normal limits, and repeated peripheral smears did not show eosinophilia(Figures 1-3).

DISCUSSION

Eosinophilic gastroenteritis is one of the rare conditions and its etiology is poorly understood. It usually involves the gastric antrum and proximal small bowel, but rarely involves the distal gut. In about 85% of cases it is associated with eosinophilia. Classification of eosinophilic enteritis is based upon the presence of eosinophilia

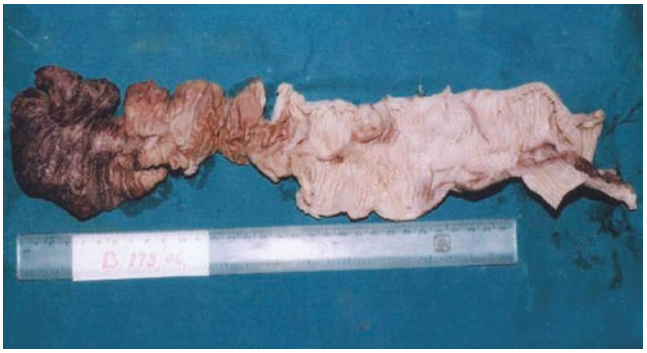

Figure 1 Ileo-ileal intussusception with a 10-cm ileal segment showing blackish brown discoloration.

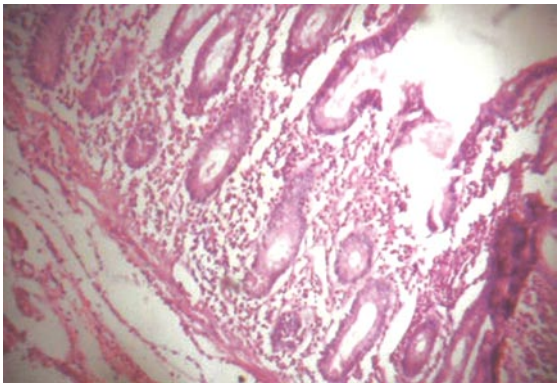

Figure 2 Microphotograph of ileum showing severe infiltration by eosinophils in all coats (HE, × 40).

or eosinopenia[1]. Clinical features depend upon the most prominent layer of visceral wall involvement by eosinophils, i.e., mucosal, muscular or serosal[2-6]. Involvement of muscularis results in obstructive symptoms and serosal involvement produces ascites. Evaluation of immunoglobulin E levels may help to detect an allergic cause. Despite all clinical factors, definitive diagnosis can only be made by histopathology confirming eosinophilic involvement of the affected area. In cases of intussusception, malignancy should always be ruled out[7].

At present, laparoscopic full thickness biopsy can be definitive in suspected cases[8]. Corticosteroid therapy is the mainstay of medical treatment. Long term follow-up is required as there are always chances of recurrence[9].

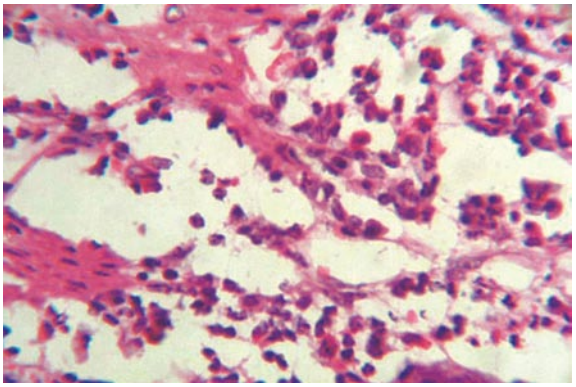

Figure 3 Microphotograph of ileum muscle coat showing severe eosinophilic infiltration (HE, × 100).

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CASE REPORT

Migrated endoclip and stone formation after cholecystectomy: A new danger of acute pancreatitis

Kemal Dolay, Halil Alis, Aliye Soylu, Gulum Altaca, Ersan Aygun

Kemal Dolay, Halil Alis, Aliye Soylu, Gulum Altaca, Ersan Aygun, Department of General Surgery1 and Gastroenterology 2, Endoscopy Unit, Bakırkoy Research and Training Hospital, Bakirkoy, Istanbul, Turkey
Correspondence to: Kemal Dolay, MD, Bakırköy Eğitim ve Araştırma Hastanesi Genel Cerrahi Kliniği Endoskopi Ünitesi Istanbul, Turkey. dolayk@yahoo.com
Telephone: +90-212-6011909 Fax: +90-212-5713278
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Abstract

Endoclip migration into the common bile duct following laparoscopic cholecystectomy (LC) is an extremely rare complication. Migrated endoclip into the common bile duct can cause obstruction, serve as a nidus for stone formation, and cause cholangitis. We report a case of obstructive jaundice and acute biliary pancreatitis due to choledocholithiasis caused by a migrated endoclip 6 mo after LC. The patient underwent early endoscopic retrograde cholangiopancreatography (ERCP) with endoscopic sphincterotomy and stone extraction.

Key words: Laparoscopic cholecystectomy; Endoclip migration; Biliary pancreatitis; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Laparoscopic cholecytectomy (LC) has become the standard approach in the treatment of benign gallbladder disease. The widespread use of LC does not increase the total incidence of postoperative complications[1]. Endoclip migration into the common bile duct was first reported by Raul *et al*[2] in 1992 as an extremely rare complication. To our knowledge, this is the first case of a migrated clip eventually resulting in acute biliary pancreatitis after LC, although choledocholithiasis due to endoclip migration into the common bile duct has been reported[1].

CASE REPORT

A 56-year-old woman with mild right upper quadrant pain for 2 d was admitted to our hospital for acute onset of severe upper abdominal pain radiating to the back accompanied with vomiting for 12 h. On physical examination, she had no fever, the sclera was icteric, and there was tenderness on the right upper quadrant of the abdomen and the epigastric area with no rigidity and rebound pain. Her amylase and lipase levels were 2066 U/L and 1980 U/L, respectively. Her liver function tests were as follows: 5 mg/dL direct bilirubin, 303 IU/L gamma glutamyl transpeptidase, 135 IU/L alkaline phosphatase, 127 mg/dL glucose, 12 g/dL blood urea nitrogen, 170 IU/L lactate dehydrogenase, 8.6 mg/dL calcium, 3.8 g/dL albumin, 66 mmHg arterial Po_2, 6800/μL white blood cells, 35% hematocrit , and 12 g/dL hemoglobin. She underwent selective noncomplicated LC 6 mo prior to this episode of acute pancreatitis.

Plain abdominal X-rays showed three endoclips in the right upper quadrant, two of them were close to each other and one was located inferiomedially to the others. Abdominal ultrasonography (US) revealed that the common bile duct was dilated to 12 mm in diameter and the pancreas was swollen. The distal common bile duct could not be evaluated adequately by US.

The patient was diagnosed as mild biliary acute pancreatitis based on the modified Imrie criteria. After medical treatment was started, ERCP performed on the second day of admittance, demonstrated a stone (12 mm in diameter) and an imbedded surgical clip at the distal part of common bile duct which was dilated to 13 mm in diameter (Figure 1A). A sphincterotomy of 15mm was performed. The extraction balloon catheter was easily burst when the stone was extracted. The stone was removed *via* a Dormia basket catheter (Figure 2A and B).

Her symptoms improved on the first day after ERCP and the serum amylase level decreased to 250 IU/L. Her amylase levels and other biochemical parameters were normal on d 2 after ERCP and she was discharged from the hospital without any complications. There was no clinical or biochemical abnormality attributable to the biliary system or pancreas during the 1-year follow-up period after ERCP.

DISCUSSION

Although the long term behavior of metal endoclips placed during LC is not clear, it is a well known-phenomenon

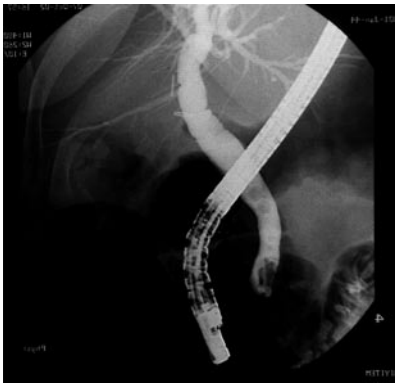

Figure 1 ERCP demonstrating a stone (12 mm in diameter) and an imbedded surgical clip at the distal part of the common bile duct.

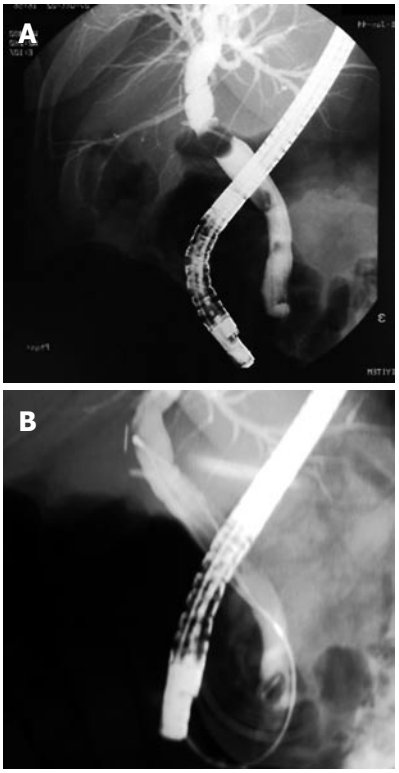

Figure 2 Placement of balloon catheter (**A**) and (**B**) Dormia basket catheter to extract the stone above the common bile duct after endoscopic sphincterotomy.

that the remained foreign materials in abdominal cavity tend to migrate towards the organs with lumens. Since stone formation within the common bile duct due to silk suture material after open cholecystectomy (OC) was first described in 1987, various foreign bodies causing nidus formation in bile ducts have been reported[3,4]. Ban *et al*[3] have divided biliary tract foreign bodies into three categories: operative residuals, penetrating missiles, and ingested items.

The first cases of choledocholithiasis due to endoclip migration into the common bile duct after OC and LC were reported by Walker *et al*[5] in 1979 and by Raoul *et al*[2] in 1992, respectively.

The true pathogenesis of migration of endoclips into the common bile duct is unclear. According to the first hypothesis, cystic duct remains patent due to ineffective clipping resulting in bilioma with bile leakage[2,6-8]. This bilioma leads to necrosis in the stump of the cystic duct by inducing chronic inflammation. The liberated clip in the necrotized cystic duct stump migrates into the common bile duct by eroding it mechanically. Presentation of the bile leakage might be subclinical, self-limited or clinically overt, which may explain why migrations occur in the early postoperative period with bile leakage.

According to the second hypothesis, the clip migrates into the ductal system by eroding the bile duct due to the local inflammation around the endoclip[6,9-12]. Short cystic duct or cystic artery, clip placement close to the common bile duct, manipulations after clipping (uncontrolled aspiration and irrigation, retraction of porta hepatis, *etc.*), local suppurative complications and ischemia on the ductal wall (excessive dissection) are risk factors for clip migration.

There have been rare reports concerning the migration of clips into the common bile duct resulting in complications other than stone formation, such as cholangitis, obstruction and stenosis of the common bile duct[6,11,13]. Cetta *et al*[14] reported a case of transient acute pancreatitis in the absence of associated stones 15 d after LC, probably due to spontaneous passage of an endoclip through the common bile duct. The case presented here is unique since stone formation and eventually acute pancreatitis occurred due to clip migration. It was reported that the time of endoclips to migrate into the bile ducts after LC is between 11 d and 6 years[2,6,7,15,16].

Absorbable clips and ultrasonic dissection without clipping in LC can prevent occurrence of clip migration and its complications[13,17]. However, these methods have not yet been used commonly by surgeons probably due to the complication and high costs of their applications. Bile duct stones associated with migration of clips after LC are usually extracted during ERCP[2,8,14]. However, when ERCP fails or in cases with additional pathology, open surgery or percutaneous transhepatic cholangioscopy can be performed[8,18].

The role of early ERCP and endoscopic sphincterotomy (ES) in the treatment of acute biliary pancreatitis is still controversial[19]. At present, early endoscopic intervention is suggested in patients with acute biliary pancreatitis when criteria for severity are met and/or there are coexistent cholangitis, jaundice, dilated common bile duct, smoldering or deteriorating clinical course[19]. In this case, we preferred early ERCP due to the presence of jaundice and dilated common bile duct.

In conclusion, endoclips after LC may migrate into the common bile duct leading to stone formation, obstruction, cholangitis, stenosis and even pancreatitis. Care should be taken during clip application, and clips should be completely squeezed by the clip applicator and unnecessary manipulations after clipping should be avoided.

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Number of monthly hits to *WJG* articles linked to PubMed surpasses 70 000

Yan Jiang

Yan Jiang, *World Journal of Gastroenterology*, The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Beijing 100025, China
Correspondence to: Yan Jiang, Assistant Editor, *World Journal of Gastroenterology*, The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center, No.62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China. submission@wjgnet.com
Telephone: +86-10-85381892 Fax: +86-10-85381893
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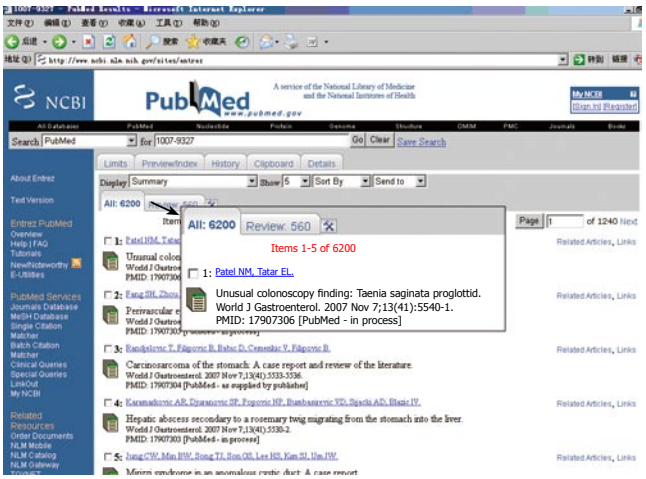

Figure 1 All 6200 articles published on *WJG* from 1998 to 2007 have been indexed by PubMed.

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International Medical Information Center, Shinanomachi Renga Bldg.35, Shinanomachi, Shinjukuku, Tokyo 160-0016, Japan

Stefano Bellentani, Professor
Fondo Studio Malattie Fegato-ONLUS, Sezione di Campogalliano, Via R. Luxemburg, 29/N, 41011 Campogalliano (MO), Italy

Filip Braet, Associate Professor
Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia

Giuseppe Chiarioni, PhD
Gastroenterological Rehabilitation Division of the University of Verona, Valeggio sul Mincio Hospital, Azienda Ospedale di Valeggio s/M, Valeggio s/M 37067, Italy

Mark G Clemens, PhD, Professor
Department of Biology, University of North Carolina at Charlotte, 9201 University City Blvd., Charlotte, NC 28223, United States

Garcia-Compean Diego, MD, Professor
Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, Mexico

Emad M El-Omar, Professor
Department of Medicine & Therapeutics, Aberdeen AB25 2ZD, United Kingdom

Francesco Feo, Professor
Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e Oncologia, Università di Sassari, Via P, Manzella 4, 07100 Sassari, Italy

Hongjin Huang, PhD
Celera Diagnostics, 1401 Harbor Bay Parkway, Alameda, California 94502, United States

Michael P Jones, MD, FACP, FACG, Associate Professor
Northwestern University Feinberg School of Medicine, Division of Gastroenterology, Suite 1400, 676 North St Clair Street, Chicago, IL 60611-2951, United States

Leonidas G Koniaris, Professor
Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

Javier San Martin
Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, Punta del Este 20100, Uruguay

James Neuberger, Professor
Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

Valerio Nobili, PhD
Liver Unit, Research Institute, Bambino Gesù Children's Hospital, S. Onofrio 4 Square, 00165 Rome, Italy

Samuel Babafemi Olaleye, PhD
Department of Physiology, University of Ibadan, Ibadan, Nigeria, Ibadan 022, Nigeria

Raffaele Pezzilli, MD
Department of Internal Medicine and Gastroenterology, Sant'Orsola-Malpighi Hospital, Via Massarenti, 9, Bologna 40138, Italy

D Mark Pritchard, PhD, FRCP
Gastroenterology of University of Liverpool, 5th Floor UCD Building, Daulby St, Liverpool L69 3GA, United Kingdom

Richard A Rippe, PhD
Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Mitsuo Shimada, Professor
Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

Tadashi Shimoyama, MD
Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan

Seng-Lai Tan, Principal Scientist
Eli Lilly and Company, Indianapolis 46285, United States

Wei Tang, MD, EngD, Assistant Professor
H-B-P Surgery Division, Artificial Organ and Transplantation Division, Department of surgery, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan

Hitoshi Togashi, Associate Professor
Department of Gastroenterology, Course of Internal Medicine and Therapeutics, Yamagata University School of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan

Yvan Vandenplas, Professor
Department of Pediatrics, AZ-VUB, Laarbeeklaan 101, Brussels 1090, Belgium

Vincent W Yang, Professor and Director
201 Whitehead Research Building, 615 Michael Street, Atlanta, GA 30322, United States

Weimin Ye, Professor
Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Box 281, SE 171 77, Stockholm, Sweden

Hiroshi Yoshida, MD
First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

Michael E Zenilman, MD
Clarence and Mary Dennis Professor and Chairman, Department of Surgery, SUNY Downstate Medical Center, Box 40, 450 Clarkson Avenue, Brooklyn, NY 11202, United States

Online Submissions: wjg.wjgnet.com
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Meeting Inflammatory Bowel Diseases 2007
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Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
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Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
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Meeting SAGES 2007 Annual Meeting -part of Surgical Spring Week
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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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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EDITORIAL

Acute hepatitis C: Prospects and challenges

Sanaa M Kamal

Sanaa M Kamal, Department of Gastroenterology and Liver Diseases, Ain Shams University, Cairo, Egypt
Correspondence to: Sanaa M Kamal, Department of Gastroenterology and Liver Diseases, Ain Shams University, Cairo, Egypt. sanaa.kamal@link.net
Telephone: +20-2-4554010 Fax: +20-2-6376372
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Abstract

More than 170 million people worldwide have chronic hepatitis C. Acute hepatitis C is rarely diagnosed because it is commonly asymptomatic. Most infected patients are unaware of their condition until the symptoms of chronic infection manifest. Treatment of acute hepatitis C is something of a paradox because spontaneous resolution is possible and many patients do not have symptoms. However, several factors provide a rationale for treating patients who have acute hepatitis C. Compared with acute hepatitis C, chronic hepatitis C is associated with a worse prognosis, the need for more intensive treatment, longer treatment duration, and a decrease in successful treatment outcomes. Conversely, early intervention is associated with improved viral eradication, using a regimen that is better tolerated, less expensive, more convenient, and of shorter duration than the currently approved combination therapies for chronic hepatitis C.

Key words: Acute hepatitis C; Therapy; Pegylated interferon

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The good news is that the annual incidence of acute hepatitis C has fallen in recent years, primarily because of effective blood and blood product screening efforts and increased education on the dangers of needle sharing. The bad news is that there are no approved therapies for treating patients with acute hepatitis C and most patients infected with the hepatitis C virus (HCV) are unaware of their exposure and remain asymptomatic during the initial stages of the infection, making diagnosis difficult and often surprising for the patient. However, there is a subset of patients - mainly healthcare workers - who have documented exposure as a result of a needle stick accident. These patients are subsequently monitored within surveillance programs to establish a documented conversion from HCV RNA-negative to HCV RNA-positive status[1]. While HCV accounts for only a minority of cases of acute hepatitis, it is the primary cause of chronic hepatitis and liver disease. With more than 170 million chronic hepatitis C patients worldwide[2] and an alarming increase in the related morbidity and mortality projected for the next decade[3], an improvement in our ability to diagnose and treat patients with acute hepatitis C would have a major impact on the prevalence of chronic hepatitis and its associated complications worldwide.

Because most acute hepatitis C patients remain undiagnosed and there is a relatively high rate of spontaneous resolution of HCV infection, little is known about the epidemiology of acute hepatitis C. Robust epidemiological data is available only for those patients who progress to chronic infection, develop symptoms, and/or seek treatment. However, what we do know about the epidemiology of acute hepatitis C is that intravenous drug and surgical/accidental exposure have been identified as the most common route of HCV transmission in most studies conducted in Western populations (Figure 1). Indeed, intravenous drug use accounted for about 25% to 54% of acute cases of hepatitis C in studies conducted in the US and throughout Europe[4-7]. In the United States alone, almost half of all HCV-positive patients aged 20 to 59 years report a history of injection drug use[8]. Transfusion-related acute hepatitis C is relatively rare; however, blood transfusion prior to 1992 remains a significant risk factor for hepatitis C infection throughout the world[8]. Sexual contact is another important route of transmission (risk factor 4% to 25%)[4-6]. Individuals with 20 or more lifetime sexual partners are at significant risk for hepatitis C in Western Countries; those participating in high risk sexual behavior are particularly susceptible because of an association with herpes simplex-type 2 infection[2,8]. In other geographic areas, social and cultural differences combine to produce a very different set of risk factors for hepatitis C. In Egypt and other African nations, illicit drug use is almost non-existent and infection mainly occurs *via* blood transfusion, or through nosocomial exposure (surgery or during circumcision)[9]. Intrafamilial exposure is also a primary route of transmission in rural Egypt[10].

Unfortunately, there is no universally agreed-upon diagnostic criteria for acute hepatitis C; a series of clinical features generally leads us to suspect acute HCV infection[11]. These 'characteristics' include an acute increase in alanine aminotransferase levels to more than

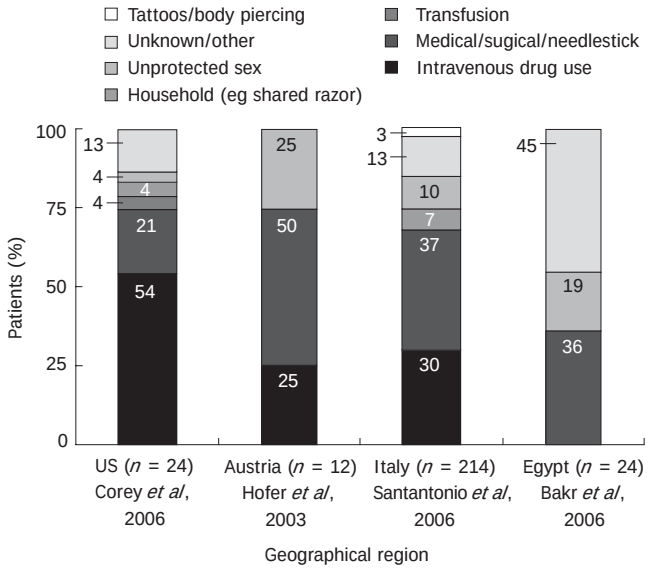

Figure 1 Acute hepatitis C sources of infection by geographic region.

10 times the upper limit of normal (with or without an increase in bilirubin) and an exposure to HCV during the previous 2-12 wk coupled with subsequent detectable HCV RNA. The few patients with acute hepatitis C who display symptoms may complain of constitutional problems or jaundice[12]; however, even among these symptomatic patients, jaundice is not always evident and slight fatigue may be the only indication that infection has taken place[13].

In many patients, HCV infection is self-limiting and spontaneously resolves before proceeding beyond the acute phase. However, establishing just how many cases resolve before reaching the chronic status has proved difficult because so few cases are actually detected. Estimates of spontaneous resolution range from 10% to 60%[1,14]; conversely, 43% to 86% of cases are thought to result in chronic infection[13]. Although these estimates vary widely, a general rule of thumb is that 20% to 26% of patients with acute hepatitis C experience spontaneous resolution[11,15]. Spontaneous resolution is most likely during the first 3-4 mo after infection; after 6 mo, it rarely occurs. So, we generally consider the first 6 mo after infection as "acute hepatitis C" and anything thereafter, "chronic hepatitis C".

Several clinical features - including the presence of symptoms and the patient's sex, immune status, and age - can serve as useful predictors of spontaneous resolution in patients with acute hepatitis C. Robust and multispecific $CD4^+$ and $CD8^+$ T cell responses are closely associated with recovery, suggesting that patients with strong immune systems have a better chance of controlling the infection[1,11]. Additionally, patients less than 40 years of age are more likely to undergo spontaneous resolution. Interestingly, infants with HCV infection have a 75%-100% chance of spontaneous resolution[11]. Conversely, patients with weakened immune systems, such as those with HIV or solid organ transplant recipients, experience more frequent progression to chronic infection[11]. Finally, self-limiting disease is also significantly more common in women than men (40% *vs* 19%, $P < 0.01$) possibly due to the facilitation of HCV clearance by an estrogen-dependent mechanism[15].

There are no currently approved treatments for acute hepatitis C; however, recently published clinical trials can be used as a basis for establishing successful management strategies. Monotherapy with standard interferon (IFN) alfa-2b or pegylated IFN (PEG-IFN) alfa-2b for 24 wk have both been shown to be effective treatments. For example, an induction regimen of IFN alfa-2b (5 MU daily for 4 wk and then 3 times weekly for 20 wk) yielded a sustained virologic response (SVR) rate of 98% in one study[16]. Similarly, 89%-95% of patients receiving PEG-IFN alfa-2b monotherapy (1.5 μg/kg per wk for 12 to 24 wk) successfully achieved viral eradication[17,18]. With both regimens, an 8-12 wk observation period between exposure to HCV and initiation of therapy is important to allow for the possibility of spontaneous resolution.

In conclusion, acute hepatitis C is a rarely encountered clinical entity. Although it is usually asymptomatic and often resolves spontaneously, it also represents an important window in the time course of this infection during which medical intervention is greeted with a high degree of success. Efforts to increase awareness, improve diagnosis, and facilitate treatment of acute hepatitis C will have far reaching implications for the management of chronic hepatitis C, where current disease management and health outcome strategies are less effective.

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REVIEW

Pathophysiology of increased intestinal permeability in obstructive jaundice

Stelios F Assimakopoulos, Chrisoula D Scopa, Constantine E Vagianos

Stelios F Assimakopoulos, Department of Internal Medicine, School of Medicine, University of Patras, Patras, Greece
Chrisoula D Scopa, Department of Pathology, School of Medicine, University of Patras, Patras, Greece
Constantine E Vagianos, 1st Surgical Department, "Saint Panteleimon" General Hospital of Nikaia, Piraeus, Greece
Correspondence to: Stelios F Assimakopoulos, MD, PhD, Department of Internal Medicine, School of Medicine, University of Patras, Vironos 18, Patras 26224, Greece. sassim@upatras.gr
Telephone: +30-2610-336656 Fax: +30-2610-993982
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Abstract

Despite advances in preoperative evaluation and postoperative care, intervention, especially surgery, for relief of obstructive jaundice still carries high morbidity and mortality rates, mainly due to sepsis and renal dysfunction. The key event in the pathophysiology of obstructive jaundice-associated complications is endotoxemia of gut origin because of intestinal barrier failure. This breakage of the gut barrier in obstructive jaundice is multi-factorial, involving disruption of the immunologic, biological and mechanical barrier. Experimental and clinical studies have shown that obstructive jaundice results in increased intestinal permeability. The mechanisms implicated in this phenomenon remain unresolved, but growing research interest during the last decade has shed light in our knowledge in the field. This review summarizes the current concepts in the pathophysiology of obstructive jaundice-induced gut barrier dysfunction, analyzing pivotal factors, such as altered intestinal tight junctions expression, oxidative stress and imbalance of enterocyte proliferation and apoptosis. Clinicians handling patients with obstructive jaundice should not neglect protecting the intestinal barrier function before, during and after intervention for the relief of this condition, which may improve their patients' outcome.

Key words: Obstructive jaundice; Intestinal barrier; Intestinal permeability; Endotoxemia; Bacterial translocation; Tight junctions; Occludin; Claudin-4; Apoptosis; Oxidative stress

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INTRODUCTION

When mechanical biliary obstruction is diagnosed, surgical, endoscopic or radiologic intervention is usually recommended. However, despite advances in preoperative evaluation and postoperative care, intervention, especially surgery, for relief of obstructive jaundice still carries high morbidity and mortality rates, mainly due to sepsis and renal dysfunction[1-3]. The concept of preoperative biliary drainage to reduce the postoperative morbidity and mortality in patients with malignant obstructive jaundice has not proved its efficacy leading to a longstanding controversy on this issue. Studies assessing the impact of endoscopic or radiologic drainage procedures prior to surgery in jaundiced patients showed high rates of complications, highlighting the role of the factor "intervention" in general in this patient population[4]. Recently, a randomized controlled multicenter clinical trial was designed to seek evidence whether or not preoperative biliary drainage should be performed in patients with obstructive jaundice due to a periampullary tumor[4].

The reasons for the high morbidity and mortality encountered in the post operative period have been attributed to impaired immune function and the high incidence of systemic endotoxemia[5-8]. In obstructive jaundice, increased intestinal permeability has been postulated to be a key factor contributing to bacterial and endotoxin translocation to mesenteric lymph nodes, portal circulation and liver[9,10]. A suppressed clearance capacity of Kupffer cells, the main hepatic macrophage population, attributed to accumulation of bile acids into liver, permits the "spillover" of endotoxin from portal into systemic circulation, with consecutive release of proinflammatory cytokines, potentially leading to the development of the so called "gut derived sepsis". Improved knowledge and understanding of the underlying pathophysiological mechanisms explaining the failure of the gut barrier in jaundiced patients may render us with better tools for prevention, treatment and patient selection.

THE GUT BARRIER STRUCTURAL AND FUNCTIONAL COMPONENTS

Nowadays, it is accepted that the gastrointestinal tract is not only a passive organ of nutrient absorption, but it

additionally displays important endocrine, immunologic, metabolic and barrier functions. The intestinal tract contains the body's largest interface between a person and his or her external environment. The complexity of its function is obvious when thinking that at the same time the intestine has to serve two opposite functions; the selective permeability of needed nutrients from the intestinal lumen into the circulation and into the internal milieu in general, and, on the other hand, the prevention of the penetration of harmful entities including microorganisms, luminal antigens, and luminal proinflammatory factors. The latter function is known as barrier function. Gut barrier function is dependent on the immune barrier, composed of locally acting factors such as, the secretory IgA, intra-mucosal lymphocytes, Payer's nodules, mesenteric lymph nodes and of the systemic host defense represented mainly by the reticuloendothelial system, the biological barrier, which is made up of normal intestinal flora responsible for colonization resistance, and the mechanical barrier, consisting of the closed-lining intestinal epithelial cells and by the capillary endothelial cells. All these components of gut barrier integrity can be affected by biliary obstruction and the absence of bile within the intestinal lumen.

THE EFFECT OF BILE ON THE GUT BARRIER

The presence of bile and bile acids in the intestinal lumen is associated with a number of positive effects, contributing to a normal gut barrier function.

Bile and the immune barrier

Experimental studies have shown that bile affects homing and distribution of T-lymphocytes in the gut-associated lymphatic tissue (GALT) and its absence results in decreased numbers of $CD4^+$ and $CD8^+$ T-lymphocytes and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressing cells in the lamina propria[11]. In addition, bile affects the size and number of B-lymphocytes in Peyer's patches. In experimental animals, the ligation of the common bile duct induced efficient apoptosis in Peyer's patch B-lymphocytes through a Toll-like receptors-2 dependent elevation of Fas expression and/or increase in sensitivity to Fas mediated apoptosis[12]. Bilirubin has been shown to impair bactericidal activity of neutrophils attenuating bacterial clearance mechanisms[13]. Bile also contains immunoglobulin A, which enhances mucosal defense either by maintaining mucosal integrity, or by binding to bacteria and viruses[14]. Circulating polymeric immunoglobulin A (IgA) binds to the secretory component (SC) on the surface of rat hepatocytes and is internalized and transported by vesicles to the canalicular membrane where the IgA-SC complex is secreted into bile. Secretion of IgA is sensitive to bile flow and the biliary secretory pathways for IgA and SC are dissociated after brief periods of cholestasis[15]. There is also evidence that specific or nonspecific antibodies contained in bile inhibit adhesion of enteric bacteria on the intestinal mucosa or inhibit bacterial endocytosis by enterocytes, thus preventing bacterial translocation[16].

Bile and the biological barrier

Bile acids have been reported to inhibit the growth of certain bacteria such as Bacteroides, Clostridia, Lactobacillus and Streptococci[17-20]. Absence of bile salts results in a disturbed intestinal bacterial balance with overgrowth of gram negative bacteria[19,21]. Bile salts have a detergent-like activity, which can make bacterial membranes permeable and can eventually lead to membrane collapse and cell damage[22]. Alternatively, bile salts are thought to prevent intestinal endotoxin and bacterial translocation by binding directly intraluminal endotoxin and bacteria, and creating poorly absorbed detergent-like complexes[23].

Bile and the mechanical barrier

In addition, bile exerts trophic effects on the intestinal mucosa, increasing villous density and inducing hypertrophy of the intestinal wall components[21,24]. *In vitro* experiments have shown that bile acids promote intestinal epithelial cell proliferation through a c-myc-dependent mechanism and protect against apoptotic cell death through activation of NF-κB[25,26]. These data support an important beneficial role of bile salts in regulation of mucosal growth and repair. Recent studies have also shown that bile is crucial for the maintenance of the integrity of enterocyte tight junctions, regulating the expression of the essential tight junction-associated proteins occludin and ZO-1, thus preserving the intestinal paracellular barrier[27,28].

INTESTINAL PERMEABILITY IN THE JAUNDICED PATIENT

Increased intestinal permeability has been postulated to be a key factor contributing to bacterial and endotoxin translocation and the pathogenesis of septic and renal complications in patients with extrahepatic biliary obstruction[29]. Beyond several experimental studies that have repeatedly demonstrated increased intestinal permeability in obstructive jaundice, this phenomenon has been confirmed in the clinical setting as well[5,29-31]. Increased intestinal permeability was evidenced in jaundiced patients either directly by the lactulose/mannitol permeability test[29,31], or indirectly by measurements of endotoxin concentrations in portal and systemic circulation[32], determination of anti-endotoxin core antibodies[31] and by multiple sampling during laparotomy in jaundiced patients, demonstrating growth of translocating bacteria of primarily enteric origin in extraintestinal sites[33]. Clinical data also demonstrate that surgical biliary decompression in obstructive jaundice exaggerates the pathophysiological disturbances and significantly increases intestinal permeability in the immediate post operative period as compared to non-surgically treated patients[5]. This probably reflects that the magnitude of an additional "trauma" in jaundiced patients is of importance and this should be considered in order not to further aggravate the patient's condition and host defense and potentially increase morbidity and mortality.

MECHANISMS OF INCREASED GUT PERMEABILITY IN OBSTRUCTIVE JAUNDICE

Intestinal permeability is determined by interactions among several barrier components including the unstirred water layer, mucosal surface hydrophobicity, the surface mucous coat, epithelial factors (especially tight junctions) and endothelial factors[34]. Each of these components has different permeability properties. However, among these factors, the intestinal epithelium consisted of the epithelial cells which are linked close to the apical surface by the tight junctions seem to be the most important in determining intestinal permeability[35]. Up to now, the mechanism of increased intestinal permeability in obstructive jaundice remains an enigma, but in the last few years experimental studies have shed light on our knowledge in the field.

In several studies, obstructive jaundice does not seem to induce dramatic morphologic changes in the intestinal mucosa on routine light microscopy[10,36], while in others non-specific findings, such as subepithelial edema, lifting of the villus and sporadic mucosal denudation with exposure of lamina propria have been documented[9,19,37]. However, ultrastructural studies on intestinal mucosa revealed certain kinds of cell disruption, represented by alterations of cellular and mitochondrial membrane[37]. In general, most studies had demonstrated that obstructive jaundice increases intestinal permeability though epithelial continuity is retained and the mechanism for this was not evident.

Tight junctions

The key event in the pathophysiology of obstructive jaundice-associated complications is gut derived endotoxemia[7]. According to its size, this molecule as well as other bacterial byproducts, could have permeated the intestinal mucosa through the paracellular pathway[38]. Therefore, our research group investigated for the first time the expression of occludin, a bona fide integral component of the tight junction, in the intestinal epithelium of jaundiced rats. The results of this study showed that intestinal mucosal barrier dysfunction in obstructive jaundice is associated with regional loss of occludin expression in the intestinal epithelium, observed mainly at the upper part of the villi[28]. Our immunohistochemical observations were confirmed by immunoblotting by other investigators, who additionally showed that obstructive jaundice leads to decreased mucosal expression of the TJ-associated protein ZO-1 as well[27]. Those researchers applying *in vitro* experiments with enterocytic monolayers incubated in the presence or absence of graded concentrations of bile showed that the alterations of intestinal tight junctions were bile mediated, while this finding was also supported *in vivo* because gavaging mice with rat bile significantly ameliorated the deleterious effects of obstructive jaundice on intestinal permeability. Also, in a current study it was shown that intestinal electrophysiological parameters in jaundiced animals, which substantially depend on intestinal TJs' integrity, were improved after oral supplementation with bile salts[39]. Further investigaton into the role of intestinal TJs alterations on gut barrier failure in obstructive jaundice demonstrated an up-regulation of claudin-4 expression in the upper part of the villi. Claudins are the only known variable elements in TJs and different expression, combination and mixing ratios of various members of the claudin family are essential in regulation of barrier properties of TJs[40]. There is evidence that the functional role of claudin-4 in the intestinal epithelium may be associated with loosening of intercellular junctions and opening of the paracellular route[41]; therefore, its overexpression is compatible with increased intestinal permeability. The key role of claudin-4 and occludin in obstructive jaundice-associated intestinal permeability alterations is further evidenced by improvement of gut mucosal barrier after restoration of their expression by regulatory peptides administration[28,42]. Apart from the role of bile deprivation, another explanation of altered intestinal occludin and claudin-4 expression in obstructive jaundice is through endotoxin-mediated mechanisms. The excessive presence of endotoxin in portal and systemic circulation stimulates a systemic inflammatory response, characterized by the release of cytokines and other proinflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1, interleukin-6, interferon-gamma (INF-γ), nitric oxide and oxygen free radicals[43]. These substances may produce injurious effects on TJs structure and function compromising intestinal epithelial barrier function[44-47]. Specifically, it has been demonstrated that TNF-α as well as INF-γ downregulate the human occludin promoter[48]. Given that increased levels of both TNF-α and INF-γ have been demonstrated in obstructive jaundice[43,49], it is tempting to speculate that these cytokines may account for occludin down-regulation. Furthermore, endotoxin reduces splachnic blood flow and disrupts intestinal microcirculation resulting in hypoxia of enterocytes and energy depletion[50]. Studies in epithelial cells monolayers have shown that adenosine triphosphate depletion induces the structural perturbation of the TJ leading to loss of the permeability barrier[51]. An additional contributory factor might be increased bacterial adherence to the enterocyte. Obstructive jaundice results in intestinal bacterial overgrowth, mainly represented by *E. coli* over growth[28]. Absence of bile deprives the gut from about 90% of secretory IgA, which normally prevents bacterial adherence to the intestinal mucosa[16]. Overgrowth of *E. coli* and lack of biliary IgA may lead to increased attachment of this bacterial strain to the intestinal mucosa. *In vitro* studies have shown that attachment of the enteropathogenic *E. coli* in intestinal epithelial cells monolayers dissociates occludin from the tight junctions, thus disrupting the paracellular barrier[52].

Cell proliferation and apoptosis

Absence of bile from the intestinal lumen is known to induce intestinal mucosal atrophy[21,53]. Epithelial homeostasis is highly dependent on the balance between cell proliferation and death, and knowledge of both factors is essential when elucidating how obstructive jaundice regulates intestinal cell turnover and mucosal cellularity. Experimental studies provided evidence of increased apoptosis of enterocytes in intestinal crypts

in parallel with decreased mitotic activity[10,54]. These cellular events occurring in intestinal crypts, where the mucosal proliferation zone exists, may explain the induction of mucosal atrophy observed in cases of biliary obstruction[54].

The responsible mechanisms of increased intestinal apoptosis could reflect primary immunologic events following BDL (apoptosis has been shown to be induced by a variety of triggers, including proinflammatory cytokines such as TNF-α, IL-1 and IL-6, or by cytotoxic T lymphocytes that act through either granzyme B or Fas receptor pathways) or a direct action of bacterial toxins[43].

It is well known that bile salts exert a potent trophic effect on the intestinal epithelium. This action is based on their mitogenic effect on the enterocytes. *In vitro* studies have shown that intestinal cells exposed to physiological concentrations of the bile salt taurodeoxycholate, within 24 h are beginning to enter into S-phase of the cell cycle, while after 6 d of exposure to bile salts, cell growth is stimulated by almost 70% relative to cells grown in the absence of bile salts[26]. The proliferative effect of taurodeoxycholate is at least partly mediated by regulation of the transcription of the proto-oncogene c-myc, which has been shown to play an important regulatory role during intestinal epithelial proliferation[26].

The significant contribution of the imbalance between cell proliferation and apoptotic death in the phenomenon of gut barrier failure in obstructive jaundice is further evidenced by the improvement of gut barrier function and the reduction of gut derived endotoxemia when factors that restore intestinal homeostasis were administered. The enhancement of intestinal permeability by glutamine may be explained by its proliferative and antiapoptotic effect on the intestinal mucosa[55-57]. Administration of intestinal trefoil agents such as Growth hormone and Insulin-like growth factor I , which act on intestinal mucosal growth, development and metabolism, significantly improved intestinal barrier function and reduced portal and systemic endotoxemia in obstructive jaundice, exerting, beyond their trophic effect, a potent antiapoptotic action on the intestinal mucosa leading to preservation of mucosal homeostasis[10]. In addition, gut regulatory peptides Bombesin and Neurotensin which have been shown to prevent gut barrier dysfunction in obstructive jaundice, exerted also a combined mitogenic and antiapoptotic effect on the intestinal mucosa[54].

Oxidative stress

Altered intestinal tight junction expression and increased intestinal apoptosis are accompanied by significant alterations of the intestinal oxidative state, which represent an additional important factor in promoting intestinal injury in obstructive jaundice[21,54,58]. Studies with experimental animals showed that obstructive jaundice induces intestinal oxidative stress evidenced, not only by increased lipid peroxidation and glutathione oxidation, but also by a general imbalance between protein or non protein thiols and protein or non protein disulfides (symmetric or mixed)[21,54,59]. Specifically, in the intestine we observed increased levels of the high oxidative stress markers of thiol redox state oxidized glutathione (GSSG), non-protein mixed disulfides (NPSSR) and protein symmetric disulfides (PSSP), accompanied by the decrease of the low oxidative stress markers glutathione (GSH), GSH:GSSG ratio and protein thiols (PSH). Our findings of increased intestinal oxidative stress in obstructive jaundice were also confirmed in the clinical setting[58]. The potential mechanisms of high intestinal oxidative stress in obstructive jaundice have been extensively reviewed previously[58,60]. Briefly, increased levels of bile acids, systemic endotoxemia and the subsequent inflammatory response[61], up-regulation of inducible nitric oxide synthase expression[62,63], increased neutrophil chemotaxis and superoxide anion generation[64] and decreased systemic levels of the antioxidant vitamin E[65], contribute to the promotion of the oxidative process in obstructive jaundice.

A question raised is whether intestinal cellular and oxidative alterations could be interrelated. It has been shown that reactive oxygen species may promote cell growth arrest, *via* a mitogen-activated protein kinases dependent pathway that alters the status of growth regulatory proteins, and apoptotic cell death, *via* a cytochrome c-mediated activation of the caspase family[66]. Thus, oxidative stress may promote intestinal apoptosis and inhibition of cell proliferation, leading to mucosal atrophy in obstructive jaundice. In addition, given that oxidative stress disrupts the TJ structural complex by modulating the assembly, localization, expression and function of their molecular components[46], this factor may underlie the altered intestinal TJ expression and increased permeability in obstructive jaundice. The interrelation of oxidative stress with intestinal cellular alterations in extrahepatic cholestasis is also supported by the fact that the oral administration of the antioxidant GSH preserves the intestinal mucosal redox state and prevents intestinal histological and electrophysiological changes[39]. In line with this observation, in a recent work[59], we demonstrated that administration of different antioxidant substances (N-acetyl-cysteine, allopurinol, α-tocopherol) in ten days' cholestatic rats, induces a significant antioxidant action in the intestine, mediated by a certain influence profile on the thiol redox state by each substance, leading to improvement of intestinal barrier function and prevention of endotoxemia. In addition, administration of gut regulatory peptides bombesin and neurotensin in experimentally jaundiced rats, induces a potent antioxidant effect on the intestine, preventing all the mentioned intestinal cellular alterations (apoptosis, inhibition of cell proliferation, altered TJ expression) and improving intestinal mucosal barrier function[28,42,54]. Taken together all these data suggest that intestinal oxidative stress and the thiol redox state are important factors in the promotion of the deleterious effects of obstructive jaundice on the anatomical and functional integrity of the intestinal mucosa.

The pathophysiology of obstructive jaundice-induced gut barrier failure, endotoxemia and systemic complications, is schematically presented in Figure 1.

CONCLUSION

Clinicians handling patients with obstructive jaundice

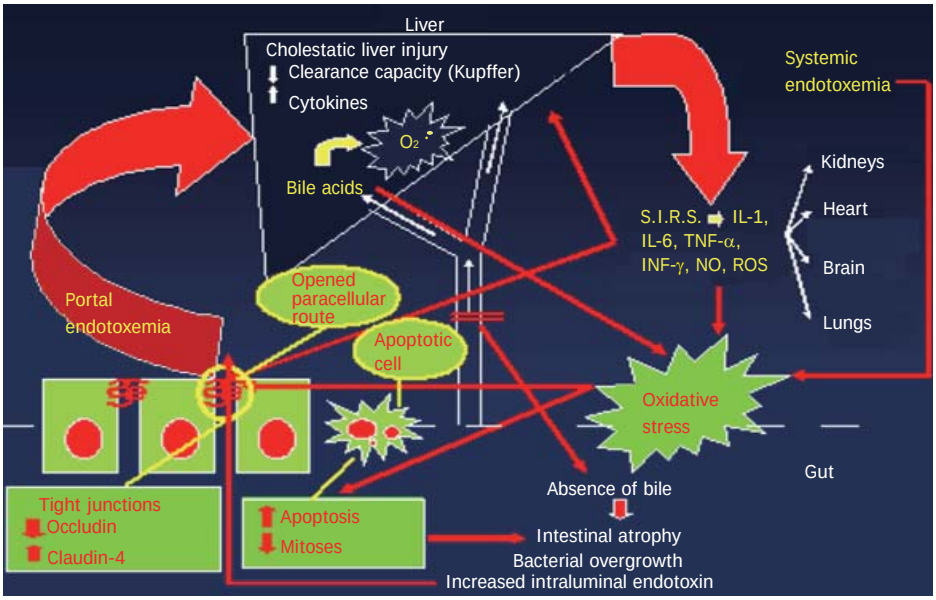

Figure 1 Pathophysiology of obstructive jaundice-induced gut barrier failure, endotoxemia and systemic complications. Absence of intraluminal bile deprives the gut from its bacteriostatic, endotoxin-neutralizing and mucosal-trophic effect leading to increased intestinal bacterial and endotoxin load and mucosal atrophy. These alterations promote bacterial and endotoxin translocation into portal circulation and subsequently, through a decreased clearance capacity of Kupffer cells because of cholestasis, into systemic circulation. Systemic endotoxemia activates a systemic inflammatory response, which is associated with dysfunction of remote organs, while it further aggravates intestinal barrier dysfunction and cholestatic liver injury. Endotoxemia, cytokinemia and increased bile acids concentrations represent important promoters of reactive oxygen species formation in diverse organs, encompassing the intestine. Increased intestinal oxidative stress in obstructive jaundice, contributes to induction of apoptosis and inhibition of cell proliferation in intestinal crypts, leading to mucosal atrophy. In parallel, intestinal oxidative stress, endotoxemia, systemic release of inflammatory mediators and absence of intraluminal bile, disrupts the integrity of enterocytes' tight junctions by altering the expression of their molecular components. As a consequence the intestinal paracellular route opens, contributing to further escape of endotoxin from the intestinal lumen into portal circulation, thus leading to a vicious cycle.

should not neglect protecting the intestinal barrier function before, during and after intervention for the relief of this condition, because failure of the intestinal barrier with consequent endotoxemia and the systemic inflammatory response may lead to serious and even life threatening complications. In this context, minimization of the additional surgical trauma, antibiotic prophylaxis, adequate fluid replacement to prevent visceral-microcirculatory disturbances, enteral nutrition to improve microcirculation, prevent mucosal atrophy and provide important nutrients for enterocytes and lactulose administration to reduce the incidence of endotoxemia are well demonstrated strategies. Growing research interest in this field has shed enough light in the pathophysiology of intestinal failure in obstructive jaundice demonstrating that the breakage of gut barrier is multi-factorial, involving disruption of the immunologic, biological and mechanical barrier. Altered intestinal tight junctions expression, oxidative stress and imbalance of cell proliferation and apoptosis may play a key role in gut permeability alterations in cases of biliary obstruction. Future studies focused on the pharmacological modulation of these factors may lead to a better control of intestinal permeability not only in obstructive jaundice, but also in diverse clinical states which may be complicated by gut-derived sepsis.

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, Professor and Sharon DeMorrow, PhD, *Series Editor*

Genetic and epigenetic changes associated with cholangiocarcinoma: From DNA methylation to microRNAs

Monique Stutes, Steven Tran, Sharon DeMorrow

Monique Stutes, Sharon DeMorrow, Division of Research and Education Scott and White Hospital, Temple, Texas 76504, United States
Steven Tran, Sharon DeMorrow, Department of Medicine, Texas A&M Health Science Center, College of Medicine, Temple, Texas 76504, United States
Supported by a NIH mentored award (KO1 DK078532) as well as a grant award from Scott & White Hospital
Correspondence to: Sharon DeMorrow, PhD, Division of Research and Education, Scott & White Hospital, Department of Internal Medicine, Texas A&M Health Science Center, Medical Research Building, 702 SW H.K Dodgen Loop, Temple, TX 76504, United States. demorrow@medicine.tamhsc.edu
Telephone: +1-254-7246240 Fax: +1-254-7248070
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Abstract

Cholangiocarcinomas are malignant epithelial liver tumors arising from the intra- and extra-hepatic bile ducts. Little is known about the molecular development of this disease, and very few effective treatment options are available. Thus, prognosis is poor. Genetic and epigenetic changes play an integral role in the neoplastic transformation of human cells to their malignant counterparts. This review summarizes some of the more prevalent genetic alterations (by microRNA expression) and epigenetic changes (hypermethylation of specific gene promoters) that are thought to contribute to the carcinogenic process in cholangiocarcinoma.

Key words: Promoter regions; CpG islands; Tumor suppressor; Oncogene; Inflammation

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INTRODUCTION

Neoplastic transformation of normal cells into their malignant counterparts often requires a series of genetic changes. These changes can range from "simple" mutations in the genes themselves that ultimately lead to loss-of-function or gain-of-function changes in key genes that are responsible for the control of apoptosis and cell cycle progression respectively, to more complex changes in non-protein factors (e.g. RNA and DNA) that regulate the control of specific gene expression[1-4]. These more complex changes can be classified as either genetic or epigenetic changes[1,3,4]. For the purpose of this review, we will define and limit the "complex" genetic changes as being any changes that occur in non-coding RNAs and epigenetic changes in the modification of the promoter regions of the genes themselves (e.g. hyper- and hypomethylation)[3-6].

Non-coding RNAs

Non-coding RNAs have recently been recognized as gene-specific regulators and therefore are similar in function to transcription factors. These RNAs can regulate every stage of gene expression, including transcription, mRNA stability and translation[5,7]. A role for these RNAs in the neoplastic transformation of cells is emerging[4]. A major sub-type of non-coding RNA is the microRNAs, which are small RNA molecules encoded in the genomes of plants and animals[5]. These highly conserved, about 21-mer RNAs regulate the expression of specific target genes by binding to the 3'-untranslated regions of mRNAs[7]. Each microRNA is thought to regulate multiple genes and since hundreds of microRNA molecules are predicted[6], the relative importance of these molecules in the control of gene expression is potentially massive. Several research groups have provided evidence that microRNAs may act as key regulators of processes as diverse as early development[8], cell proliferation and cell death[9], apoptosis and fat metabolism[10], and cell differentiation[11,12]. In addition, recent studies suggest a possible link between microRNAs and various cancers, including chronic lymphocytic leukemia[13], colonic adenocarcinoma[14], Burkitt's Lymphoma[15] and cholangiocarcinoma[16-18].

Epigenetic changes

Epigenetic gene silencing refers to non-mutational gene inactivation that can be passed from parental cells to daughter cells[1]. The addition of methyl groups to cytosine residues in CpG dinucleotides in DNA is a biochemical modification that meets this requirement, referred to as hypermethylation[1]. Genes carrying epimutations

cause morphological phenotypes to be transmitted from generation to generation, not based on any alteration in the coding sequence of the relevant genes, but instead caused by CpG (or CpNpG) hypermethylation of their promoter sequences[2]. Methylation patterns in mammalian cells are regulated by DNA methyltransferases[19], which transfer a methyl group to the cytosine portion of the CpG dinucleotide[19]. This allows for the binding of methyl-specific DNA-binding proteins such as MeCP1 or MeCP2 to regulatory elements, which in turn represses transcription[20]. These binding proteins can then attract histone deacetylases, which then remodel chromatin into highly repressed states[20]. Hypermethylation has been shown to play an important role in the progression of tumor growth in almost all types of cancer[21].

Recent data suggest that both genetic and epigenetic changes are required for transformation, promotion and progression of cholangiocarcinoma[16,17,22-26], a deadly cancer of the cells lining the intra- and extra-hepatic biliary tract[27,28]. This type of cancer is increasing in its incidence worldwide, with no effective treatment options[29,30]. Therefore, understanding the molecular events associated with the neoplastic transformation of cholangiocytes to cholangiocarcinoma may aid in the development of improved therapeutic strategies. This review will summarize our current understanding of the most prevalent genetic and epigenetic changes associated with cholangiocarcinoma.

THE REGULATION OF TUMOR SUPPRESSOR GENES AND CELL CYCLE INHIBITORS

Methylation

The hypermethylation and inactivation of a number of cell cycle inhibitors have been shown to occur in cholangiocarcinomas. The most well-characterized of these epigenetic changes is in the $p16^{INK4a}$ gene, which has been described in up to 83% of cholangiocarcinomas[23,26]. This gene is responsible for binding to the cyclin dependent kinase 4 (CDK4) and inhibits its ability to interact with cyclin D1[31]. In the absence of $p16^{INK4a}$ activity, such as after promoter methylation, CDK4 binds to cyclin D1, which subsequently leads to unchecked entry into the S phase of the cell cycle[31]. There also appears to be increased incidence of hypermethylation of the related $p14^{ARF}$ occurring in 25% of cholangiocarcinoma samples studied[25]. The $p14^{ARF}$ gene normally prevents p53 degradation and hence cell-cycle arrest[32], which constitutes another checkpoint that may be lost in cholangiocarcinoma[33]. In addition to the above-mentioned genes, many other cell cycle entry inhibitors have been shown to be hypermethylated. These include $p16^{INK4b}$ (50% of tumors studied)[26] and 14-3-3 sigma (59.5% of tumors studied)[34].

In addition, the expression of many tumor suppressor genes is repressed in cholangiocarcinomas[24]. The most striking of which is Semaphorin3B, which was found to be methylated in 100% of the cholangiocarcinoma cases studied[24]. RassF1A[26] and p73[26] are also hypermethylated and suppressed in 65.3% and 36.1% of cholangiocarcinoma cases studied respectively.

Taken together, the epigenetic silencing of a vast array of tumor suppressor genes and inhibitors of cell cycle progression obviously plays an important role in the initiation and progression of cholangiocarcinoma. Inactivation of these genes allows cells to avoid apoptosis and to proliferate unchecked.

microRNA

To date, the data concerning microRNA regulation of cell cycle and/or apoptotic genes is sparse. Recently Meng *et al* showed that miR-141 was highly overexpressed in malignant cholangiocytes[16]. Using a bioinformatics approach, a predicted target of miR-141 was the CLOCK gene, which regulates circadian rhythms and can act as a tumor suppressor[16]. Inhibiting miR-141 effectively increased CLOCK protein expression in cholangiocarcinoma cells[16]. Another microRNA species that was found to be overexpressed in malignant cholangiocytes was miR-200b[16]. The target gene for this was predicted to be the protein tyrosine phosphatase non-receptor type 12 (PTPN12), the dysregulation of which may contribute to tumor cell survival and oncogenesis[16]. Similarly, the expression of miR-21 was overexpressed in cholangiocarcinoma, which effectively blocks the expression of the tumor suppressor gene PTEN[16].

Conversely, other microRNA species have been identified as being downregulated in cholangiocarcinoma compared to non-malignant cholangiocytes. miR-29b expression was suppressed in the cholangiocarcinoma cell line KMCH as well as in approximately 33% of human cholangiocarcinoma samples[18]. Enforced miR-29b overexpression in cholangiocarcinoma cells effectively reduced the expression of Mcl-1, an anti-apoptotic protein of the Bcl-2 family[18] and sensitized cholangiocarcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand cytotoxicity, suggesting that the suppression of miR-29b expression found in cholangiocarcinoma allows the overexpression of Mcl-1 and can ultimately lead to the resistance of cholangiocarcinoma to cell death[18]. Another microRNA that is downregulated in cholangiocarcinoma is miR-370[17]. Interestingly, the expression of this particular microRNA has been shown to be under tight epigenetic regulation by hypermethylation[17]. One of the targets for miR-370 is the oncogene mitogen-activated protein kinase kinase kinase 8 (MAP3K8)[17], thus MAP3K8 is upregulated in cholangiocarcinoma cell lines as well as in tumor cell xenografts *in vivo*[17].

THE REGULATION OF GENES INVOLVED IN DNA REPAIR

Methylation

Inherited mutations that affect DNA repair genes are strongly associated with high cancer risks in humans. A number of the DNA repair genes have been shown to be hypermethylated in cholangiocarcinoma. Hypermethylation of the hMLH1 mismatch repair gene promoter has been shown to occur in up to 23.6%

of cholangiocarcinomas[26], which has previously been revealed to lead to microsatellite instability in other tumor types[35]. Another gene, O6-methylguanine-DNA methyltransferase (MGMT), is silenced in up to 33% of cholangiocarcinoma tumors studied[26]. This gene is an important suicide enzyme involved in the defense against O6-alkylating endogenous metabolites and environmental carcinogens. Interestingly, transcriptional repression of MGMT was associated with the accumulation of GC-AT transitional mutations in the p53 gene and, to a lesser extent, the k-*ras* gene in cholangiocarcinoma[26,36]. Lastly, the glutathione S-transferase P1 (GSTP1) gene, which inactivates electrophilic carcinogens by conjugation with glutathione is hypermethylated in cholangiocarcinoma, occurring in 6% to 34% of cases studied[26,34]. Taken together, the hypermethylation of genes responsible for DNA repair appears to be an important step in the carcinogenic process of cholangiocarcinoma.

microRNA

To our knowledge, and to date, no genes involved in DNA repair have been identified as targets of transcriptional control by microRNA in cholangiocarcinoma.

THE REGULATION OF GENES INVOLVED IN INFLAMMATION

Methylation

Chronic inflammation, such as those seen in various cholestatic liver diseases, is a recognized risk factor for the development of cholangiocarcinoma[27,28]. Therefore, it follows that certain mediators of the inflammatory process may be integral in the carcinogenic processes associated with neoplastic transformation of cholangiocytes[37-39]. Indeed, sustained overexpression of the cytokine interleukin-6 (IL-6) has been demonstrated to have an integral role in cholangiocarcinoma biology[38,39]. This aberrant overexpression of IL-6 has recently been shown to be as a consequence of the epigenetic silencing of the suppressor of cytokine signaling 3 (SOCS-3)[40]. SOCS-3 promoter methylation was observed in a subset of cholangiocarcinoma samples as well as in a number cholangiocarcinoma cell lines[40]. Enforced overexpression of SOCS-3 in these cell lines effectively reduced the IL-6-mediated signal transduction cascade[40]. Therefore the loss of this negative regulator of IL-6 in cholangiocarcinoma may contribute to the inflated expression and activity of inflammatory molecules seen in cholangiocarcinoma.

A downstream consequence of aberrant IL-6 expression may be the further hypermethylation of the promoter regions of a number of critical target genes in cholangiocarcinoma. IL-6 has been shown to regulate the enzyme activity of one of the DNA methyltransferases responsible for the hypermethylation of promoter regions[41]. In cholangiocarcinoma cells, IL-6 overexpression resulted in the altered promoter methylation of a number of genes including the epidermal growth factor receptor (EGFR)[42]. EGFR promoter methylation was decreased and gene and protein expression increased by IL-6[42], suggesting that the epigenetic regulation of gene expression by the inflated IL-6 expression seen in cholangiocarcinoma can contribute to tumor progression by altering the expression of growth regulatory pathways, such as those involving EGFR[42].

microRNA

In addition to changes in promoter methylation, aberrant IL-6 expression in cholangiocarcinoma also has implications on microRNA expression and function. Enforced IL-6 overexpression in human cholangiocarcinoma cell lines significantly increased the expression of several members of the let-7 family of microRNAs[22]. Expression of let-7a contributes to the survival effects that are attributed to IL-6 over-expression[22]. A putative target of let-7a microRNA is the gene neurofibromatosis 2 (NF2)[22], which is a negative regulator of Stat-3[43]. Thus, overexpression of IL-6 in cholangiocarcinoma and subsequent upregulation of let-7a decreased the expression of NF2, thereby removing the negative regulation of Stat-3[22]. Constitutive activation of Stat-3 has been implicated in a number of cancers and is thought to be responsible for the IL-6-mediated survival signaling[22].

THE REGULATION OF GENES INVOLVED IN CELL ADHESION AND INVASION

Methylation

E-Cadherin is a calcium-dependent cell adhesion molecule that suppresses metastatic processes and tumor cell invasion[44-46]. In cholangiocarcinoma, methylation of the E-cadherin promoter occurred in up to 48% of all samples studied[25,26,34,47]. Downregulation of this gene by epigenetic changes has been reported in a number of other cancers[44,45], and re-expression can be induced by treatment with a demethylating agent in cholangiocarcinoma[48].

microRNA

To our knowledge, no genes that are involved in cell adhesion or the metastatic process associated with cholangiocarcinoma biology have been shown to be regulated by microRNAs.

CONCLUSION

In conclusion, from the epigenetic changes summarized in this review, it is obvious that epigenetic silencing as well as genetic regulation by microRNAs play a very important role in the neoplastic transformation of cholangiocytes to their malignant counterparts. A summary of the changes in specific promoter hypermethylation and microRNA can be found in Tables 1 and 2 respectively. We acknowledge that this summary is far from complete and that more target genes are being described regularly. Further analysis and characterization of these genetic and epigenetic changes, as well as the potential interplay between hypermethylation and microRNA expression will aid in the identification of therapeutic targets for the design of treatment strategies to combat this deadly malignancy.

Table 1 Summary of genes subject to epigenetic silencing during the course of cholangiocarcinoma tumor progression

Gene	Incidence of methylation in CCH (%)	Function	Reference
$p16^{INK4a}$	14-50	Cell cycle regulator	[26,34,47]
$p14^{ARF}$	38	Cell cycle regulator	[26]
$p15^{INK4b}$	12-50	Cell cycle regulator	[26,47]
14-3-3 sigma	59.50	Cell cycle regulator	[34]
Semaphorin3B	100	Tumor suppressor	[24]
p73	36	Tumor suppressor	[26]
RassF1A	26-65	Tumor suppressor	[26,47]
hMLH1	25	DNA mismatch repair	[26]
MGMT	11-33	Methyl transferase	[26,34]
GSTP1	6-34	Inactivation of carcinogens	[26,34]
SOCS-3	ND	Inhibits inflammation	[40]
EGFR	ND	Growth factor	[42]
E-cadherin	43	Cell adhesion	[25,26,34,47]

ND: Not determined.

Table 2 MicroRNAs known to be changed in cholangiocarcinoma

MicroRNA	Target gene	Function	Direction changed in CCH	Reference
miR-141	CLOCK	Circadian rhythm	Increased	[16]
miR-200b	PTPN12	Tumor suppressor	Increased	[16]
miR-21	PTEN	Tumor suppressor	Increased	[16]
miR-29b	Mcl-1	Anti-apoptotic gene	Decreased	[18]
miR-370	MAP3K8	Oncogene	Decreased	[17]
let-7a	NF2	Negative regulator of inflammation	Increased	[22]

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, Professor and Sharon DeMorrow, PhD, *Series Editor*

AKT and ERK1/2 signaling in intrahepatic cholangiocarcinoma

KJ Schmitz, H Lang, J Wohlschlaeger, GC Sotiropoulos, H Reis, KW Schmid, HA Baba

KJ Schmitz, J Wohlschlaeger, H Reis, KW Schmid, HA Baba, Institute of Pathology and Neuropathology, University Hospital of Essen, University Duisburg-Essen, Germany
H Lang, GC Sotiropoulos, Department of General, Visceral and Transplantation Surgery, University Hospital of Essen, University Duisburg-Essen, Germany
H Lang, KW Schmid, Members of the West German Cancer Center, Germany
Correspondence to: HA Baba, Institute of Pathology and Neuropathology, D-45147 Essen, Hufelandstr. 55, Germany. hideo.baba@uk-essen.de
Telephone: +49-201-7233577 Fax: +49-201-7233378
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Abstract

Intrahepatic cholangiocarcinomas (ICC) are neoplasms that originate from cholangiocytes and can occur at any level of the biliary tree. Surgical resection is the current therapy of choice for this highly aggressive cancer. However, the 5-year survival still is poor, with high recurrence rates. Due to the intrahepatic growth a significant proportion of patients present with advanced disease and are not candidates for curative surgery or transplantation. The existing palliative options are of limited benefit and there is a great necessity for novel therapeutic options. In this article we review the role of the phosphoinositide 3-kinase(PI3K)/ AKT and extracellular regulated kinase (ERK) signaling pathways in ICC and present new data on the prognostic value of these protein kinases. Finally, we discuss future upcoming therapeutic options based on targeting these signaling pathways.

Key words: Extra cellular regulated kinases; Cholangiocarcinoma; Prognosis; Oncology; Immunohistochemistry

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INTRODUCTION

Cholangiocarcinomas (CCC) are rare malignant tumors arising from the biliary tract. According to their anatomic location they can be categorized as either intrahepatic or extrahepatic. Although intrahepatic cholangiocarcinoma (ICC) is less frequent than extrahepatic carcinoma, within malignant liver tumors it ranks second after hepatocellular carcinoma. The incidence of cholangiocarcinoma differs considerably in different geographic regions, with the incidence highest in Southeast Asia[1]. In western countries ICC accounts for about 10% of primary liver malignancies with increasing incidence. Established risk factors for development of cholangiocarcinoma are liver fluke infestation especially with opisthorchis viverini, primary sclerosing cholangitis, hepatolithiasis, anomalous biliary-pancreatic malformation, choledochal cysts, thorotrast exposure, liver cirrhosis and hepatitis C infection[2]. However, most cholangiocarcinomas arise in the absence of known underlying risk factors.

The prognosis of ICC is very poor. Due to its intrahepatic localization, symptoms occur late in the course of the disease and patients often present with an advanced tumor. Most patients exhibit a median survival of less than 9 mo after diagnosis. To date, complete resection has been the only curative therapy. Recent trends are to advocate accurate preoperative staging with an aggressive surgical approach to achieve complete tumor resection[2,3]. In the majority of patients ICC presents at an advanced stage or patients have associated co-morbidity that preclude surgery. This patient group needs adequate palliation such as chemotherapy. Biliary decompression, which can be achieved by surgery, radiology or endoscopy, is an additional palliative treatment option. However, since ICC rarely cause biliary congestion, these additional palliative options are rarely used.

The importance of an optimal preoperative assessment of resectability and the lack of potent optional (adjuvant) therapeutic approaches, emphasize the necessity of novel prognostic and predictive parameters. This review focuses on the relevance of two central biological signaling pathways - namely the AKT and ERK1/2 pathway - in ICC. The development of phospho-specific antibodies for immunohistochemistry allows the evaluation of signaling activity of these pathways in pathological specimens. Phosphorylation of proteins either on specific tyrosine or serine residues is a posttranslational event modulating the activity of many key signaling molecules in the cell including AKT and ERK1/2[4]. We recently demonstrated AKT and ERK phosphorylation as an independent prognostic parameter in breast cancer and colorectal cancer, respectively[5,6]. Others found an association of AKT

and ERK1/2 with decreased survival in various human malignomas including breast cancer, colorectal cancer, pancreatic cancer, malignant melanoma, leukemia and mucoepidermoid cancer[5,7-10]. This review describes the biological background of AKT and ERK in ICC, presents our own new data, analyses the potential prognostic relevance and discusses upcoming therapeutic options.

GENERAL BIOLOGY OF THE AKT AND ERK SIGNALING PATHWAYS

AKT/PKB (Protein kinase B)

AKT is a central player in the regulation of metabolism, cell survival, motility, cell cycle progression and transcription. AKT is a serine/threonine kinase and its activation is induced by phosphorylation mediated by Phosphoinositide (PI) 3-kinase (PI3K) in association with tyrosine kinase receptors. The AKT family comprises three mammalian isoforms, PKBα, PKBβ and PKBγ (AKT1, AKT2 and AKT3, respectively), which are products from different genes and share a conserved structure. AKT includes three functional domains: an N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal regulatory domain. PI3K is localized upstream of the AKT kinase and is essential for the activation of AKT. PI3K and thereby AKT are activated upon (1) autophosphorylation of receptor tyrosine kinases induced by ligands (such as growth factors), (2) activation of cytokine receptors, (3) stimulation of G-Protein coupled receptors, or (4) activation of integrin signaling[11,12]. Upon PI3K mediated generation of the second messenger PtdIns (3,4,5) P_3 AKT is translocated from the cytoplasm to the plasma membrane. Once recruited to the membrane, AKT is activated by a phosphoinositide-dependent kinase 1 and 2 (PDK1, PDK2) dependent multistep process that results in the phosphorylation of both Threonine 308 and Serine 473 residues necessary for full AKT activation. Due to the great number of downstream substrates AKT kinase modulates a variety of central cellular processes as summarized in Figure 1.

ERK (extracellular regulated kinases)

ERK1/2 - also referred to as p44 and p42 MAP kinases - are ubiquitously expressed kinases and represent one component of the three mitogen activated protein kinases (MAPK) cascades that are activated by an enormous array of stimuli. The MAPK pathways phosphorylate and activate numerous proteins, including transcription factors, cytoskeletal proteins, kinases and other enzymes. Each of the three MAPK pathways contains a three-tiered kinase cascade comprising a MAP kinase kinase kinase (synonyms: MAPKKK, MAP3K, MEKK or MKKK), a MAP kinase kinase (synonyms: MPKK, MAP2K, MEK or MKK) and the MAPK. This review focuses on ERK1 and ERK2 that form a part of the MAPK module containing Raf MAPKKKs (A-Raf- B-Raf, C-Raf/Raf1) and the MEK1/MEK2 MAPKKs. All MAPKs are activated by dual phosphorylation of the conserved threonine and tyrosine residues. ERK1 and ERK2 are induced by stimuli such as tyrosine receptor kinases or G-protein coupled receptors. This leads to activation of Ras, which then triggers a complex network including the activation of Raf isoforms. ERK1/2 exhibit a variety of substrates including several key transcription factors (Figure 1). Depending upon the intensity and duration of stimulation ERK1/2 can result in proliferation of differentiation[13].

TARGETS OF THE AKT AND ERK PATHWAY

Both AKT and ERK mediate their effect *via* several substrates, which may be localized in the nuclei or in the cytoplasm. AKT can potentially phosphorylate over 9000 substrates in mammalian cells including typical cytoplasmic as well as nuclear proteins. Thus, AKT activity not surprisingly can be detected in both the nucleus and the cytoplasm[14]. When interpreting immunohistochemically stained tissue slides, it is important to keep the subcellular localization of activated AKT or ERK in mind. Moreover it seems as if both kinases show different subcellular localizations in various human carcinomas. In a colon cancer study, we showed pAKT immuno-localization in the nucleus and cytoplasm[6], in contrast to our results in ICC with a restricted immunoreactivity in the cytoplasm.

Cytosolic substrates for ERK include several pathway components involved in ERK negative feedback regulation. Multiple residues on SOS (son of sevenless homolog) are phosphorylated by ERK following growth factor stimulation. SOS phosphorylation destabilizes the SOS-Grb2 complex, eliminating SOS recruitment to the plasma membrane and interfering with Ras activation of the ERK pathway. It has also been proposed that negative feedback by ERK occurs through direct phosphorylation of the epidermal growth factor EGF receptor at Thr669[15].

ERK1 and ERK2 regulate transcription indirectly by phosphorylating the 90 kDa ribosomal protein S6 kinases (RSKs), a family of broadly expressed Ser/Thr kinases activated in response to mitogenic stimuli, including growth factors and tumor-promoting phorbol esters[16]. Active RSKs appear to play a major role in transcriptional regulation, translocating to the nucleus and phosphorylating such factors as the product of proto-oncogene *c-fos* at Ser362, serum response factor (SRF) at Ser103, and cyclic AMP response element-binding protein (CREB) at Ser133[17,18]. Recent studies revealed a spatial control of ERK signaling by Sef (similar expression of FGF), a recently identified inhibitor, whose action mechanisms are not fully defined. Sef acts as a molecular switch for ERK signaling by specifically inhibiting nuclear translocation of ERK without inhibiting its activity in the cytoplasm[19]. Further studies are necessary to elucidate the regulatory mechanisms of Sef.

Upon phosphorylation, nuclear translocation of ERK1 and ERK2 is critical for both gene expression and DNA replication induced by growth factors. Probably the best-characterized transcription factor substrates of ERKs are ternary complex factors (TCFs) including Elk-1, which is directly phosphorylated by ERK1 and ERK2 at multiple sites[20]. Upon complex formation with SRF, phosphorylated TCFs transcriptionally activate the numerous mitogen-inducible genes regulated by serum response elements

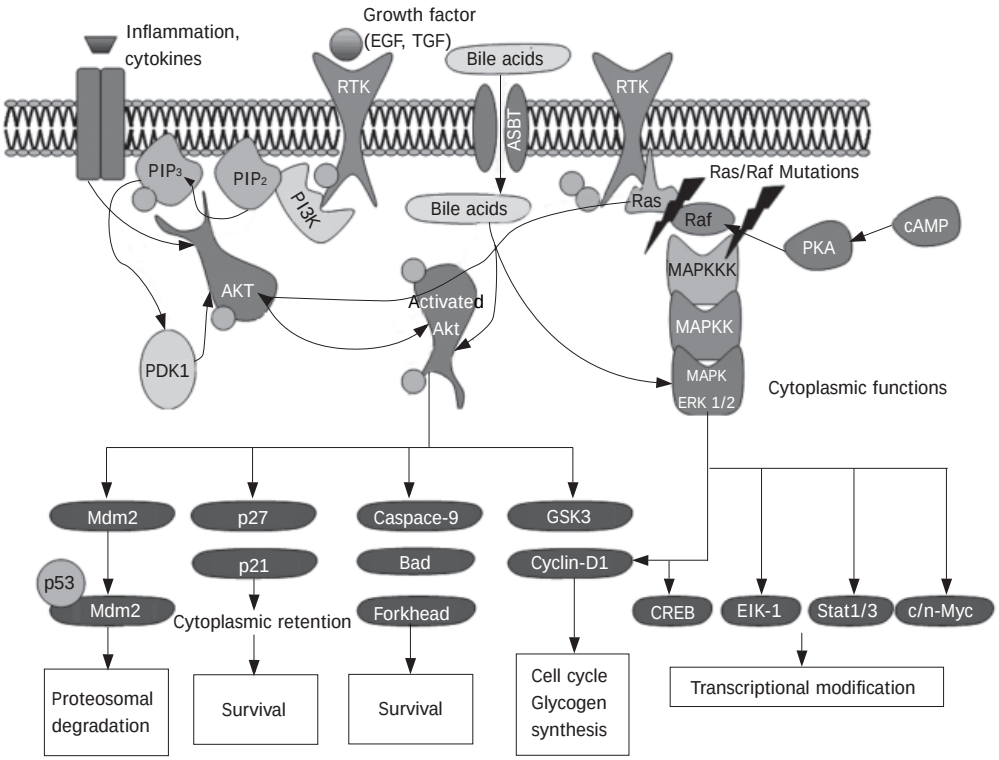

Figure 1 Overview of the AKT and ERK signaling pathway.

(SREs)[21]. Another direct target of ERK, at least *in vitro*, is the product of proto-oncogene c-*myc*, a short-lived transcription factor involved in multiple aspects of growth control[22].

AKT may mediate its functions both in the cytoplasm and in the nucleus. AKT phosphorylates a great variety of substrates involved in the regulation of key cellular functions including cell growth and survival, glucose metabolism and protein translation. Most of the well-known targets are located in the cytoplasm but it is noteworthy that many of the substrates of AKT are proteins that function in the nucleus. Some relevant cytoplasmic targets of AKT are GSK3 (glycogen synthase kinase), IRS-1 (insulin receptor substrate-1), PDE-3B (phosphodiesterase-3B), BAD, human caspase 9, Forkhead and NF-B transcription factors, BRCA1, MDM2 (murine double minute), mTOR (mammalian target of rapamycin), eNOS (endothelial nitric oxide synthase), Raf protein kinase and $p21^{Cip/Waf1}$. Several of these targets such as MDM2 translocate to the nucleus upon activation *via* AKT [23-28]. Similar to ERK1/2, immunohistochemical analysis of pAKT in various human cancers shows both cytoplasmic and nuclear immunoreactivity. *In vitro* studies showed that AKT is activated by membrane localization and later translocated to the cytosol and nucleus[29]. The function of nuclear activated AKT is not yet fully understood. Among the nuclear substrates there are fatty acid synthase[30], estrogen and androgen hormone receptors[31,32] and transcriptional factors of the FOXO family[33]. All these factors are supposed to increase cell survival.

SPECIAL ASPECTS OF AKT AND ERK IN CHOLANGIOCARCINOMA

Both the AKT and ERK pathways can be activated *via* several growth factors/survival factors, stimulation of G-protein-coupled receptors, or activation of integrin signaling. Due to the great array of activators relevant to different human cancers, we will focus on those activators that are likely to be specific for ICC.

Activation by growth factors

Both the AKT and the ERK pathway are inducible *via* growth factor stimulation. It is known that a subset of ICC exhibit overexpression of the EGF and HER-2 receptor[34]. Indeed, in our study group 21.3% (13/61) of ICC showed strong EGFR overexpression, which was confirmed by others[35]. Due to the relatively large subset of ICC with EGF or HER-2 expression activation of these ligands might be relevant for the progression of ICC.

Activation by cytokine receptors

A well-known risk factor for the development of cholangiocarcinoma is chronic inflammation. Chronically inflamed biliary epithelium is exposed to cytokines and chemokines. Indeed, cholangiocarcinoma cells constitutively secrete Interleukin 6 (IL-6), which is supposed to be a pivotal cytokine for cholangiocarcinogenesis[36-38]. A recent paper found ERK1/2 and AKT in cholangiocarcinoma cell lines to be activated *via* the cytokine receptor C-X-C motif chemokine receptor 4 (CXCR4) *via* the CXC chemokine ligand 12 (CXCL12). Thus, inflammation signals mediated by cytokine receptors are capable of inducing the ERK and AKT pathway[39].

Activation by oncogenes

It is crucial to know that AKT and ERK can be induced by mutation of oncogenes such as *Ras* and *Braf*. *Ras* is frequently mutated in many tumors, and is associated with constitutive activation of the ERK1/2 MAPKs. A

recent study showed that in contrast to hepatocellular carcinoma (HCC), *K-ras* and *Braf* mutations are a frequent event in cholangiocarcinoma[40]. In fact 22% of the cholangiocarcinoma analysed exhibited a *K-ras* mutation and 45% a *B-Raf* mutation, respectively.

Since Ras and Braf proteins are members of the Ras/Raf/MAPKKK/MAPKK/MAPK pathway, activating mutations of *K-ras* and *B-raf* should result in increased activation of MAPK. Indeed it was shown, that tumors harboring a *B-raf* mutation exhibited stronger ERK1/2 immunostaining[40]. *K-ras* mutations are a frequent event in colorectal cancer and we recently demonstrated a consecutive activation of the ERK pathway in cancers with mutated *K-ras*[6]. These data indicate that frequent disruption of alterations in the Ras/Raf/MAPKKK/MAPKK/MAPK pathway, either by *Ras* or *Braf* mutations, may play a central role in cholangiocellular carcinogenesis.

Activation by bile acids

Cholangiocytes are exposed to high concentrations of bile acids. It is known that bile acids after cellular uptake by apical sodium bile acid cotransporter (ASBT), are capable of altering the AKT and ERK signaling pathways[41]. Moreover, the EGFR-pathway appears to be activated by extracellular bile acids *via* its ligand transforming growth factor TGF-alpha in cholangiocytes[42] and the human cholangiocarcinoma cell line[43]. The EGFR pathway, once activated initiates several signaling cascades, including the AKT and ERK pathways.

ERK AND AKT PATHWAYS AND PROGNOSIS

Little is known about the prognostic relevance of the ERK and AKT pathways in intrahepatic cholangiocarcinoma. In fact, there is only one study that was based on a small cohort of 24 samples that does not discriminate between extra- and intrahepatic cholangiocarcinoma[44]. Thus, we aimed to elucidate the clinical impact of activated AKT and ERK pathways in a large and homogenous cohort of solely intrahepatic cholangiocarcinoma.

We would like to present new data derived from a large study of 62 intrahepatic cholangiocarcinomas. Between 1998 and 2006 a total of 62 patients with a mean age of 58 ± 11.5 years were available for this study. The study comprised consecutive patients who underwent surgery for liver resection. Patients solely undergoing an explorative laparatomy without subsequent resection or with hilar cholangiocarcinoma, gallbladder carcinoma or mixed hepato/-CCC were excluded from the study. The diagnosis of ICC was based on histology by examination of the resected liver specimen.

IMMUNOHISTOCHEMISTRY (IHC)

Phospho-AKT and Phospho-ERK

Immunostaining with pAKT (1/2/3) serine 473 was carried out with a monoclonal anti-phospho-AKT antibody (Cell Signaling Technology, Beverly, Massachusetts, USA). Subsequent to antibody retrieval, the primary antibodies were incubated for 30 min at 1:20 dilution. Antibody detection was performed with peroxidase-conjugated streptavidin and diaminobenzidine as chromogen. Tumors were classified according to their cytoplasmic staining intensity: negative (0), moderate (1) and strong (2).

Monoclonal phospho-p44/42 MAPK antibody (threonine 202/ tyrosine 204; Cell Signaling Technology, Beverly, Massachusetts, USA) was used at a 1:100 dilution. Tumor cells with strong specific immunostaining, independent of the amount of stained cells, were scored as strongly positive (2+). Tumors exhibiting a detectable but faint immunostaining were scored as weak (1+), whereas tumors with a minimal, hardly detectable or missing staining pattern were classified as negative (0).

EGFR

Immunostaining with EGFR was carried out with a monoclonal anti-EGFR antibody (Zymed Laboratories Inc, CA, USA). The primary antibodies were incubated for 30 min at 1:100 dilution. Antibody demonstration was achieved using the commercially available anti-mouse IgG detection kit (EnVision, DakoCytomation, Carpenteria, CA, USA). Classification was performed following the guidelines of PharmDx^{Tm} (DakoCytomation). Tumor samples lacking immunostaining were classified as negative (0), whereas the remaining were classified into 1+, 2+ or 3+ depending on the level of immunoreactivity.

Ki67 Immunostaining, TUNEL

The growth fraction was determined as previously described[45]. *In situ* DNA fragmentation was established using the terminal desoxyribonucleotide transferase (TdT)-mediated dUTP nick end labelling technique (TUNEL) as previously described[45].

Statistical analysis

Immunostainings were assessed by two of the authors in a blind-trial fashion without knowledge of the clinical outcome. Interobserver agreement[46] of pERK and pAKT was substantial (kappa = 0.78 and 0.63). Relationships between ordinal parameters were investigated using two tailed χ^2 analysis (or Fisher's exact test where patient numbers were small). Overall survival (OS) curves were estimated using the Kaplan-Meier method, and any differences in the survival curves were compared by the log-rank test.

IHC of pAKT, pERK and EGFR

Immunostaining for pAKT was localized to the cytoplasm of tumor cells. Twenty two patients (37.3%) were classified as negative, 24 (40.7%) as moderately positive and 13 (22%) as strongly positive. Immunostaining of pERK exhibited a specific nuclear and cytoplasmatic staining pattern. Representative pAKT and pERK immunostainings are shown in Figure 2. In all, 26 (41.9%) tumors lacked pERK immunostaining, 19 (30.6%) tumors exhibited a moderate staining intensity and 17 (27.4%) tumors were classified as strongly positive. Immunostaining of EGFR was localized to the membrane of the tumor cells. Due to the lack of paraffin material, two cases were excluded from EGFR-

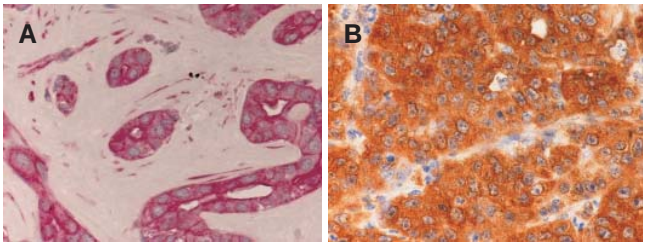

Figure 2 Light micrograph displaying strong phospho-ERK1/2 (**A**) and strong phospho-AKT (**B**) expression as analyzed by immunohistochemistry in intrahepatic cholangiocarcinoma (x 400).

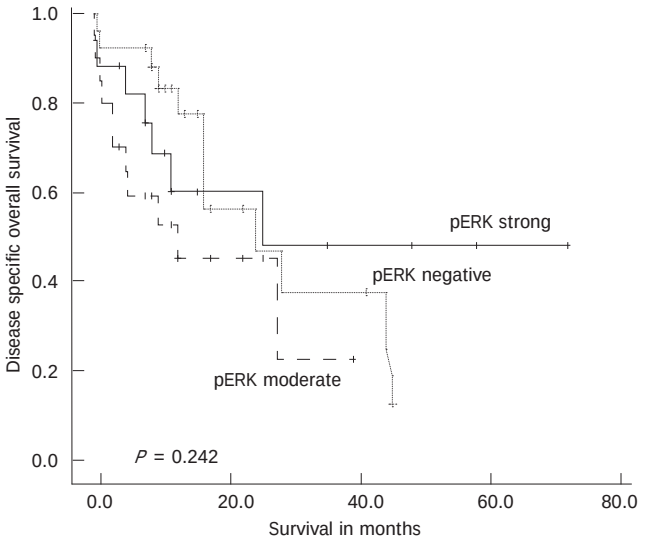

Figure 3 Kaplan-Meier survival plot for disease specific overall survival in the complete series of 62 intrahepatic cholangiocarcinoma in relation to pERK immunostaining intensity. Log-rank test: $P = 0.242$.

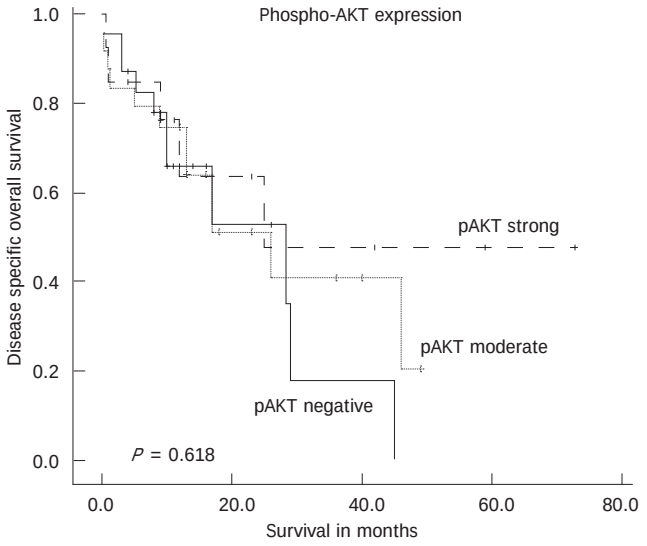

Figure 4 Kaplan-Meier survival plot for disease specific overall survival in the complete series of 59 intrahepatic cholangiocarcinoma in relation to pAKT immunostaining intensity. Log-rank test: $P = 0.618$.

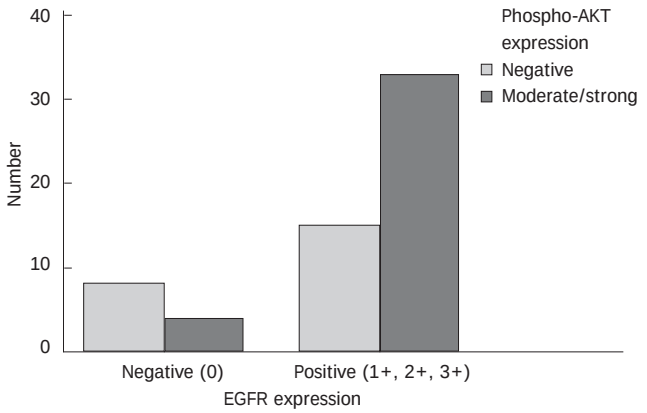

Figure 5 Intrahepatic cholangiocarcinoma with positive EGFR expression exhibit significantly more frequent AKT activation (χ^2 analysis: $P = 0.028$).

IHC. In all, 12 (19.7 %) tumors were classified as negative, 19 (31.1 %) as 1+, 17 (28.9%) as 2+ and 13 (21.3%) as 3+. χ^2 analysis revealed that EGFR-positive tumors (1+, 2+, 3+) had a statistically significant association with AKT activation ($P = 0.028$; Figure 5) but not with ERK activation. Kaplan-Meier survival analysis did not reveal a prognostic relevance of pAKT, pERK (Figures 3 and 4) or EGFR expression nor were any of the parameters associated with apoptosis or growth fraction (data not shown).

CONCLUSION

The ERK and AKT pathways are of central relevance and can induce growth factor independent growth, insensitivity to apoptotic signals, ability to invade and alter response to chemotherapeutic drugs. The fact that mutations of the Ras/Raf/MAPKKK/MAPKK/MAPK pathway occur in more than 60% of cholangiocarcinomas indicates the importance of these pathways for the carcinogenesis of cholangiocarcinomas and highlights new strategies in the treatment of this disease[47]. Moreover, the finding of a relatively large subset of ICC presenting overexpression of growth factors such as EGFR and HER-2/neu support the notion, that these growth factors play a relevant role in the progression of ICC. Our results support the notion that the AKT but not the ERK1/2 pathway is induced by EGFR overexpression, since we found a coexpression of pAKT and EGFR in ICC. However, neither ERK1/2 nor AKT activation influenced patient survival in this large series of ICC. Thus, both kinases may not serve as potential prognostic markers in this highly aggressive disease. Our results are in contrast to a previous study suggesting AKT as a favourable prognostic parameter in cholangiocarcinoma[44]. This study by Javle *et al* did not discriminate between intrahepatic and extrahepatic cholangiocarcinoma and is based on a small cohort with 24 patients; thus, the data derived should be interpreted with caution. The main advantage of the present study is the large number of patients ($n = 62$) in combination with a high homogeneity of this series composed of consecutively resected ICC.

Both the AKT and ERK pathways are topics of preclinical and clinical trials. A promising candidate is the multikinase inhibitor Sorafenib, which inhibits several

kinases including the Raf kinase, an upstream activator of the ERK pathway[48,49]. A recent study published the results of a phase Ⅱ study of sorafenib in patients with advanced hepatocellular carcinoma. It was demonstrated that HCC patients with higher pERK baseline levels had a longer time to progression following treatment with sorafenib thus pointing towards the possible relevance of activated pERK as an useful biomarker in HCC[48,49]. With regard to carcinomas of the biliary system the results of a phase Ⅱ study of sorafenib in patients with unresectable or metastastic gallbladder carcinoma or cholangiocarcinoma have been presented in Abstract form[50]. Sorafenib as a single agent did not result in a clinically significant objective response rate in patients with gallbladder and cholangiocarcinoma, but demonstrated an impact on survival that may be comparable to commonly used chemotherapy regimens. These promising data point towards novel therapeutic options utilizing multikinase inhibitors such as Sorafenib. In addition, the inhibition of upstream activators of the AKT and ERK pathways such as EGFR and HER-2 might also constitute novel therapeutic approaches. The results in a small number of patients showed that cetuximab, a monoclonal antibody against EGFR, is well tolerated and provides good palliative effects in advanced cholangiocarcinoma[51].

Despite the widely acknowledged potential of AKT inhibitors as anticancer therapy, few have made it to clinical trials. Wortmannin and LY294002 may have limited clinical utility owing to their lack of specificity, associated adverse effects, poor pharmacology, and poor solubility[52,53]. *In vivo* use of LY294002 in mice has been associated with many adverse effects, including death[54]. Furthermore, in mice, Wortmannin induces liver and bone marrow toxicity, and LY294002 can induce dermatitis and inhibit growth. Therefore, although Wortmannin and LY294002 inhibit the PI-3K/AKT pathway, their drawbacks raise doubts about their suitability as leading candidates for additional drug development.

Currently studies aimed at testing the clinical relevance of AKT inhibitors such as VQD-002, [Triciribine (TCN-P)] which is a tricyclic nucleoside that inhibits activated AKT, are being conducted. For example, the MD Anderson Cancer Center, Houston, TX (USA), and the H. Lee Moffitt Cancer Center, Tampa, FL (USA) have an ongoing Phase Ⅰ/Ⅱa clinical trial with VQD-002 in ovarian, pancreatic, breast, and colorectal cancer patients with tumors that over-express AKT (http://www.vioquestpharm.com/content/VQ0A002_about.html). Regarding the MAPK pathway, oral MEK inhibitors such as CI-1040 are currently being tested in a multicenter phase Ⅱ study in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer[55].

In addition to the above-mentioned kinases, another potential therapeutic target is worth mentioning. Against the background of NSAIDs, recent studies have evaluated selective COX-inhibitors for their effect on cell growth and invasion of cholangiocarcinoma cells *in vitro* and in nude mice[56-60]. Treatment with COX-2 inhibitors resulted in induced apoptosis and inhibited proliferation. In a recent study we demonstrated the independent prognostic value of immunohistochemical COX-2 protein expression in resected ICC[45], thereby offering a further potential additional adjuvant therapeutic approach with COX-2 inhibitors facilitating an optimised therapeutic strategy. Moreover COX-2 may serve as a target for chemoprevention in high-risk patients.

Significant progress has been made in understanding this disease, and patients are being diagnosed earlier at specialized centers. Moreover, an optimized preoperative assessment of resectability as well as an aggressive intraoperative approach to achieve complete tumor resection might increase long-term survival[3]. However, a significant proportion of patients present with advanced disease and are not candidates for curative surgery. The palliative options, mainly consisting of chemotherapy, are of limited benefit, as cholangiocarcinomas respond poorly to existing therapies. Therefore, further clinical and preclinical trials are necessary in order to develop novel therapeutic options based on new tumor targets such as AKT, ERK and EGFR. Although the activation of these pathways did not show an impact on survival in ICC - at least in this study - in contrast to many other human carcinomas, an interruption of these pathways or associated signaling molecules by specific inhibitors might, nevertheless, have favorable effects on long-term survival for this highly aggressive cancer.

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TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, Professor and Sharon DeMorrow, PhD, *Series Editor*

JAK-STAT pathway in carcinogenesis: Is it relevant to cholangiocarcinoma progression?

Olga V Smirnova, Tatiana Yu Ostroukhova, Roman L Bogorad

Olga V Smirnova, Tatiana Yu Ostroukhova, Laboratory of Endocrinology, Biological Faculty, M.V. Lomonosov Moscow State University, Moscow, Russia
Roman L Bogorad, Inserm, U845, Paris France; Université Paris Descartes, Faculté de Médecine René Descartes, Centre de Recherche Croissance et Signalisation, Equipe PRL, GH and tumours, Site Necker, Paris, F-75015, France
Correspondence to: Olga V Smirnova, PhD, DSc, Laboratory of Endocrinology, Biological Faculty, M.V. Lomonosov Moscow State University, Leninskie Gory, 1/12, 119992, Moscow, Russia. smirnova_ov@mail.ru
Telephone: +7-495-9393678 Fax: +7-495-9394309
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Abstract

The features of JAK-STAT signaling in liver cells are discussed in the current review. The role of this signaling cascade in carcinogenesis is accentuated. The possible involvement of this pathway and alteration of its elements are compared for normal cholangiocytes, cholangiocarcinoma predisposition and development. Prolactin and interleukin-6 are described in detail as the best studied examples. In addition, the non-classical nuclear translocation of cytokine receptors is discussed in terms of its possible implication to cholangiocarcinoma development.

Key words: Cholangiocarcinoma; Janus tyrosine Kinases; Signal Transducers and Activators of Transcription; Prolactin; Interleukin-6; Cytokine receptors; Receptor tyrosine kinases

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INTRODUCTION

The signaling pathway of Janus tyrosine Kinases-Signal Transducers and Activators of Transcription (JAK-STAT) is activated by a variety of hormones (prolactin, growth hormone, leptin, erythropoietin), as well as cytokines, and growth factors *via* their receptors. JAK-STAT signaling participates in the regulation of cell proliferation, differentiation, survival, motility, and apoptosis in different organs including liver[1]. During the last decades, the data on cytokine/growth factor receptors expression and functions in different liver cell types (hepatocytes, cholangiocytes, Kupffer and stellate cells) have rapidly grown. It highlights the importance of JAK-STAT signaling in normal liver physiology and pathology. Moreover, deregulation of this pathway is closely associated with tumorigenesis.

Cholangiocarcinoma is a malignancy of the biliary epithelium with the worst prognosis among other liver tumors. It is relatively rare, but it frequently progressively increases. Molecular cholangiocarcinoma markers are mainly nonspecific and require further investigation. The main risk factors for cholangiocarcinoma development are hyperplasia of bile duct epithelia induced by bile duct obstruction, hepatolithiasis, inflammation, fibrosis, and some other factors[2-4]. Though the role of the JAK-STAT cascade is poorly deciphered both in normal cholangiocytes and in cholangiocarcinoma cells, a number of papers have been published recently where the role of prolactin (Prl), growth hormone (GH) and interleukin-6 (IL-6) and expression of corresponding receptors in cholangiocytes has been shown[5-7]. Expression of these receptors undergoes essential changes under hyperplasia of bile duct epithelia induced by bile duct obstruction, thus the role of the JAK-STAT pathway should attract further investigations in this area.

PRINCIPLES OF JAK-STAT SIGNALING

The main site for JAK-STAT signaling initiation is the cellular membrane; however, internalization pathways have been recently shown to be also important for the generation of comprehensive biological effect and sustaining signal transduction. Among other intracellular targets of receptor-JAK-STAT the cascade nucleus plays a key role[8-10]. Mitochondrial targeting of some cytokine receptors (GH receptor) has also been described[11].

The initial steps of signaling cascade activation by hormone (cytokine) are still intensively studied, but for many of them an interaction between signaling molecules and preformed receptor dimers leads to rotation of intercellular parts of receptor and subsequent activation of receptor-associated JAKs[12] followed by STAT docking on receptor and its phosphorylation, dimerization, and nuclear translocation (Figure 1).

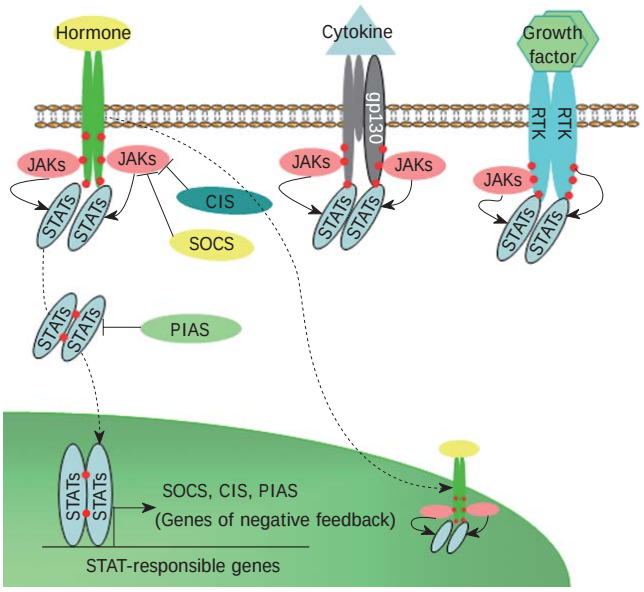

Figure 1 Schematic representation of JAK-STAT signaling induced by hormones, cytokines, and growth factors. RTK: Receptors tyrosine kinases; JAK: Jannus kinase; STA: Signal transducer and activator of transcription; SOCS: Suppressor of cytokine signaling; CIS: Cytokine induced suppressor; PIAS: Protein inhibitors of activated STATs. Solid arrows - phosphorylation; Dashed arrows - nuclear translocation; Blunt arrows - inhibition; Red circles - phosphorylated tyrosines.

Different JAKs (JAK1, JAK2, JAK3, and Tyk2) and STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) participate in JAK-STAT signal transduction. STAT3 and STAT5 are expressed in many cell types, activated by various hormones, cytokines and growth factors and play a role in different biological responses, whereas other STAT proteins (STAT1, 2, 4 and 6) are revealed mainly in specific cell types and participate predominantly in host defense mechanisms[13,14]. Phosphorylated STAT dimers can bind to STAT-responsive elements in the promoters of various genes, resulting in modulation of their transcription. STATs often act on gene transcription in cooperation or competition with NF-κB, AP-1, and a glucocorticoid receptor due to close co-localization of correspondent binding sites[15-17] accomplishing a crosstalk with other signaling cascades.

Suppressors of cytokine signaling (SOCS) proteins are able to inhibit JAK-STAT signaling. SOCS1 and SOCS3 are the most potent and broadly distributed suppressors with similar inhibition effects on gp130 related, Prl, and GH signaling. Promoter regions of SOCS1 and SOCS3 genes possess functional STAT binding elements and activation of JAK-STAT signaling induces rapid up-regulation of SOCS proteins by STAT-dependent pathways. In accordance with negative feedback mechanism JAK-STAT signaling is inhibited by SOCS proteins association with catalytic domains of JAKs as well as by SOCS binding to phosphorylated tyrosine residues of cytokine, GH, Prl, leptin, and erythropoietin (Epo) receptors which act as docking sites for downstream signaling[18-20]. STAT activation can also be inhibited by direct interaction with protein inhibitors of activated STAT proteins (PIAS)[21].

In some cases activation of STATs occurs independently of JAKs and is involved in receptor tyrosine kinase signaling. This activation is induced by direct interaction with receptor tyrosine kinases or by subsequent downstream signaling with implication of other kinases such as Src. For example, STAT3 is activated in response to stimulation by EGF, HGF, CSF-1, PDGF and other growth factors; and their corresponding receptor tyrosine kinases (ErbB1, ErbB-2, c-met, CSF-1, PDGF receptors, and others) possess a common STAT3 docking motif in their cytoplasmic domains. STAT5a phosphorylation is induced by EGF[13,22,23].

JAK-STAT SIGNALING AND CARCINOGENESIS

Altered activation of JAK-STAT signaling has been shown in multiple solid tumors and leukemia[24-26]. In different experimental models tumorigenesis is associated with increased expression and/or activity of JAK1, 2[27,28] or STAT3, 5[29]. A constitutively active mutant of STAT3 has been shown to transform rat fibroblasts[30], and the increased expression of wild type STAT5 in the lymphoid lineage induced T cell leukemia in mice[31]. A constitutively active mutant of JAK2 has been identified in patients with polycythaemia vera[32]. Moreover, increased levels of correspondent ligands can be associated with JAK-STAT induced carcinogenesis: in animal models of prostate and mammary gland hyperplasia with local overexpression of Prl[33,34]; in humans hyperprolactinemia is considered as a risk factor for breast and, probably, prostate cancer[35,36]; overexpression of GH in transgenic mice leads to increased frequency of mammary adenocarcinoma[37]. A particular and essential role for neoplasia progression of autocrine production of Prl has been revealed[34,38]. Only autocrine, but not exocrine GH, is suggested to promote neoplasia[39-41].

The role of the JAK-STAT cascade is not limited to induction of carcinogenesis: for example, the predominant role of TYK2 and STAT1 in many types of cancer is antitumorigenic *via* regulation of apoptosis[42]. Moreover, in some models JAK1 and 2 also play an antitumorigenic role[43]. Prl induced activation of JAK2 prevents breast cancer cells mesenchymal transition and acts as an invasion suppressor[44,45]. Reduced phosphorylation of STAT5 in breast cancer highlights patients with a worse prognosis of disease development[46].

CHOLANGIOCARCINOMA DEVELOPMENT AND JAK-STAT SIGNALING

A number of signaling molecules acting via the JAK-STAT pathway participate in regulation of bile duct cell functions. Among them, Prl and IL-6 (activation of complete JAK-STAT cascade), and EGF, HGF (activation of STAT *via* receptor tyrosine kinases) have their receptors in human and animal cholangiocarcinoma tissues and cell lines. Other JAK-STAT signaling molecules involved in the development of other cancer types have not been yet investigated.

Since the development of cholangiocarcinoma is closely related with obstructive, fibrotic, cirrhotic, and inflammation

changes of biliary epithelium leading to its hyperplasia or dysplasia, it is reasonable to compare the peculiarity of JAK-STAT signaling induced by different ligands in normal cholangiocytes, bile duct cells proliferating under conditions of different hepatic pathologies considered as a predisposition to cholangiocarcinoma development, and in cholangiocarcinoma cells.

HORMONAL INDUCTION OF JAK-STAT SIGNALING AND CHOLANGIOCARCINOMA DEVELOPMENT

Prolactin signaling

Prl is a multifunctional pituitary hormone that also works as a locally produced cytokine. Prl regulates differentiation, proliferation, water-salt balance in different cell types including many ductal structures (such as ductal systems of mammary, lacrimal, submaxillary, prostate glands, pancreas, and kidney) and possesses high immunomodulatory activity[47,48]. Prl participates in the stimulation of rat bile duct cell proliferation and cholangiocyte regulation of bile water salt balance[49,50].

Prl receptor (PrlR) exists in several isoforms with a tissue specific ratio of expression. Long and short rat PrlR isoforms are the result of alternative splicing of cytoplasmic domain exons of a single gene. Several intermediate PrlR isoforms with varying cytoplasmic domains have been found in human normal and tumor tissues. The short PrlR isoform has been suggested to negatively regulate activity of the long isoform by heterodimerization. JAK-STAT pathway with predominant usage of JAK2 and STAT5 proteins is the main signaling pathway activated by Prl. STAT1 and STAT3 proteins may also be involved in Prl signaling with nuclear translocation of their homo- and heterodimers. SOCS1, 2, and 3 attenuate Prl signaling[47,48,51-53].

Prolactin JAK-STAT signaling and their alterations under conditions of cholangiocarcinoma predisposition and development

Systemic Prl levels are elevated under different liver pathologies associated with increased cholangiocarcinoma incidents[54-56], while local Prl production by the cholangiocarcinoma cell line (RS-1) is observed only in a few samples (Ostroukhova & Smirnova, unpublished observations).

Normal animal and human bile duct cells express PrlR messenger RNA and protein at a relatively low level. Contrary to hepatocytes where short PrlR isoform predominates, normal rat bile duct cells express a predominantly long receptor isoform that is similar for such Prl target tissues, such as the uterus, pituitary, and mammary gland[7,57].

Hyperplasia of bile duct epithelia in rats induced by bile duct obstruction or normally occurring in immature animals as a risk factor of cholangiocarcinoma development is characterized by sharp elevation of cholangiocyte PrlR expression in both males and females. Similarly, obstructive human liver diseases with jaundice development and

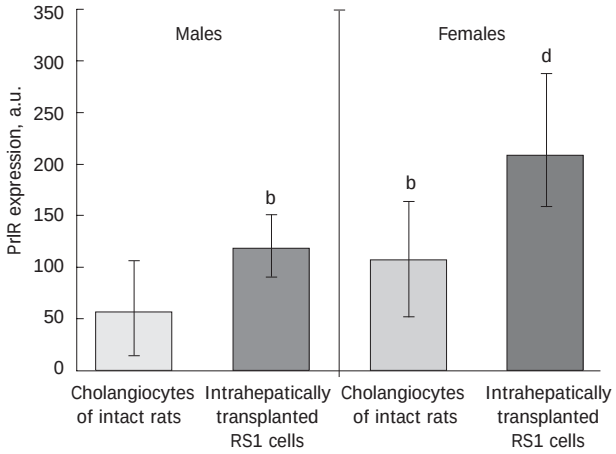

Figure 2 Increasing of prolactin receptor expression in rat liver transplanted RS-1 cholangiocarcinoma cell line. a.u, arbitrary units of intensity of PrlR expression. Medians, upper and lower quartiles. $^{b}P < 0.001$, *vs* intact males, $^{d}P < 0.001$, *vs* intact females.

enrichment of bile ducts are accompanied by a pronounced increase in cholangiocyte PrlR expression as compared with mild cholestasis without jaundice with limited affect on bile duct cell proliferating activity[57]. Obstructive conditions induce both dramatic elevation of the long receptor isoform and an increase in short isoform expression in bile duct cells. The level and isoform ratio of cholangiocyte PrlR in normal and obstructive conditions are only slightly dependent on gonadal hormones and independent of Prl[7,49,57].

Human cholangiocarcinoma samples are characterized by high PrlR expression significantly exceeding PrlR expression in cholangiocytes and hepatocytes under conditions of non-tumor liver diseases (Ostroukhova & Smirnova, unpublished observations). Intrahepatic transplantation of RS1 cholangiocarcinoma cells induces significant elevation of tumor cell PrlR expression (Figure 2) as compared both with subcutaneous tumor transplants and cholangiocytes of normal animals[58].

In cholangiocarcinoma cells the isoforms ratio is similar to the ratio observed in cholangiocytes proliferating under obstructive conditions. Surprisingly, relatively high levels of PrlR in transplantable cholangiocarcinoma have been revealed not only in female recipients, but also in male counterparts under conditions of relatively low levels of both Prl and estrogens[58]. Thus, it seems possible that the main regulators of PrlR expression in cholangiocarcinoma differ from sex hormones and may be connected with tumor derived autocrine/paracrine factors. Such unusual regulation may be an early event of cholangiocyte dysplasia since sharp elevation of PrlR expression in both males and females primarily occurs in bile duct cells proliferating in response to bile duct obstruction[7,49].

Prolactin - estrogen cross-talk and cholangiocarcinoma development

Prl as a female sex hormone acts in close connection with estrogens. In addition to systemic positive regulation of Prl production by estrogens and vise versa, a cross-talk between these hormones has been shown at the level of target tissues.

Prl stimulates both alpha and beta mRNA estrogen receptor expression in some tissues and putative STAT5α and STAT5β response elements have been identified in their promoters[59].

Estradiol *via* estrogen receptor (ER) alpha activates transcriptional expression of human PrlR through promoter Ⅲ that functions in different tissues including liver. Estrogens increase and tamoxifen reduces expression of Prl receptor mRNA in breast tumors and some other tissues[60-62]. Estrogens participate in regulation of downstream Prl signaling using different mechanisms. Estrogens can attenuate JAK-STAT signaling by Prl. Estrogens *via* ER α up-regulate SOCS2 expression thus diminishing JAK-STAT signaling. ER alpha activation could diminish PrlR signaling *via* direct interaction with STAT5α which leads to reduced STAT5α nuclear translocation. On the contrary, nongenomic action of estrogens that requires a ligand-binding domain of the membrane estrogen receptor alpha leads to rapid induction of STAT3 and STAT5 phosphorylation, their nuclear translocation, and transcriptional activity[61,63-66].

All these facts are very important taking into account that both PrlR and ERs are sharply increased in human cholangiocarcinoma samples and cell lines. While normal cholangiocytes express extremely low ERs, cholangiocarcinoma cells are characterized by intensive cytoplasmic and nuclear expression of ERα and ERβ as well as increased ERα/ERβ ratio that is typical for other cancer types. Besides, these estrogens potentiate and tamoxifen attenuates proliferation of human cholangiocarcinoma cell lines *in vitro*[4,6]. As in the case of PrlR, high level of ER expression in cholangiocarcinoma have been revealed not only in females, but also in males[67]. Based on both cytoplasmic and nuclear ER localization in cholangiocarcinoma cells it seems possible that estrogen regulation of Prl induced tumor JAK-STAT signaling is both nuclear and nongenomic and its bottom-line effects should be investigated carefully. It is interesting that in spite of high sensitivity of cholangiocarcinoma cells to both types of female sex hormones cholangiocarcinoma development is more frequent in men (1.5-1) than in women[68]. It may be due to attenuation of Prl tumor signaling by estrogens in the case of relatively high levels of both hormones in women. In any case combined therapy with tamoxifen and bromocriptine may be perspective clinical strategy for treatment of cholangiocarcinoma.

Cross-talk of Prl with EGF signaling leading to ErbB1 and ErbB2 phosphorylation has also been found, e.g. in breast cancer cells[69]. Since both Prl receptor and ERbB1 and ErbB2 are present in cholangiocarcinoma cells it may be relevant also to cholangiocarcinoma Prl signaling.

Growth hormone (GH) JAK-STAT signaling

As in the case of Prl, JAK-STAT signaling activated by GH mainly includes JAK2 activation and STAT5 phosphorylation, though STAT1 and 3 activation are sometimes also implicated. GH induces the expression of several negative regulators of GH signaling: SOCS1, 2, and 3, CIS[70,71].

GH receptor is present in normal rat cholangiocytes and isolated cholangiocytes respond to GH stimulation by IGF-1 secretion that in turn promotes cholangiocyt proliferation. GH receptor expression is increased in rat cholangiocytes after common bile duct ligation[72]. Contrary, in total liver of mice under the same conditions and humans with obstructive cholestasis and cirrhosis, changes of GH receptor expression and IGF-1 production are opposite[73]. GH signaling in cholangiocarcinoma cells has not been yet investigated.

Leptin JAK-STAT signaling

Leptin receptor is homologous to gp130 and can activate JAK2, mainly STAT3, but also STAT5, STAT6, STAT1 proteins[74-77]. SOCS3 is a negative regulator of leptin JAK-STAT signaling[18,78,79].

Autocrine/paracrine leptin production and leptin mRNA have been shown in different types of cancer cell lines *in vitro* and in some tumors *in vivo*. Serum leptin level also increases in patients with some cancer types[76,80]. Leptin is considered an agent responsible for overcoming the antiproliferative effect of antiestrogen therapy in different cancer types. Leptin receptors have been found in different cancer types and cell lines and leptin signaling is associated with suppression of tumor cell apoptosis[13,78].

Leptin expression has been detected in liver stellate cells and its local production is increased under obstructive cholestasis conditions. Leptin acts as a direct hepatic stellate cell survival agent[81-83]. Serum leptin is also elevated after common bile duct ligation and CCl_4 induced liver fibrosis that are both risk factors for cholangiocarcinoma development[84]. The leptin receptor has been found in bile duct cells of one type of mammalian species[85]. The role of leptin induction of JAK-STAT signaling needs to be investigated in cholangiocarcinoma development, and known STAT3 activation during cholangiocarcinoma progression (see below) may be partly due to leptin signaling.

Erythropoietin (Epo) JAK-STAT signaling

Epo is produced mainly by the fetal liver and the adult kidney. Epo interaction with Epo receptor activates JAK2 and STAT5[86,87]. Due to its erythropoietic functions, Epo has been widely used for prevention and treatment of cancer-associated anemia. However, Epo proangiogenic activity can compromise its beneficial effects in cancer patients.

Expression of Epo and Epo receptors has been found in a number of nonhematopoietic tissues as well as in different types of primary tumors and tumor cell lines suggesting the generation of autocrine-paracrine growth stimulatory Epo-Epo receptor loop in cancer cells. Experimental blockade of Epo signaling results in inhibition of different tumor growth and angiogenesis with concomitant elevation of apoptotic death of tumor and vascular endothelial cells[87-90].

Epo and/or Epo receptor expression have been found in liver stromal cells and some primary hepatic tumors, and tumor cell lines as well as in Kupffer cells during regeneration[87,89-93]. Still, we have not found any data on Epo expression and signaling in human cholangiocarcinoma

and its animal models. Based on the frequency of Epo and Epo receptor expression in different types of tumor cells, a key role of the fetal liver in Epo production, a role of hepatic progenitor cells, enriched in fetal liver, in liver cancer development as well as possibility of bone marrow derived liver stem cells to differentiate into biliary cells[90,94-97] it is easy to assume that the same situation occurs in cholangiocarcinoma development. Several facts support this assumption. In an earlier study the elevation of liver Epo production after bile duct ligation has been shown[98]. Data on the possibility of tissue specific estrogen modulation of local Epo expression together with elevation of estrogen receptor expression in human cholangiocarcinoma are also of great interest in this connection[6,86,99,100].

CYTOKINE INDUCTION OF JAK-STAT SIGNALING AND CHOLANGIOCARCINOMA DEVELOPMENT

Interleukine-6 (IL-6) induced JAK-STAT signaling

IL-6 and other cytokines of this family have their own receptors, but need a common receptor subunit gp130 for signal transduction. Binding of IL-6 to its receptor leads to dimerization of gp130, resulting in activation of gp130-associated JAK kinases (JAK1, JAK2, and TYK2) and subsequent activation of STAT3 proteins forming homodimers or rarely heterodimers with STAT1. There is reciprocal negative regulation of JAK-STAT3 and MAPK signaling by IL-6[15,101,102]. SOCS3 activated by STAT3 suppresses IL-6 signaling[15,18-20].

Alterations of local and systemic IL-6 level under conditions of cholangiocarcinoma predisposition and development

Autocrine/paracrine production of IL-6 by normal liver bile duct cells as well as by cultured human intrahepatic biliary epithelial cells is very low. Bile duct obstruction triggers pronounced elevation of IL-6 mRNA and protein production by bile duct cells, which can be mediated by inflammatory conditions[103-105]. A pronounced autocrine/paracrine secretion of IL-6 by different human intrahepatic cholangiocarcinoma cell lines has been observed *in vitro*[106,107]. Obstructive changes as well as cholangiocarcinoma development are also associated with elevation of systemic plasma IL-6 level[108].

Alterations of IL-6 signaling under conditions of cholangiocarcinoma predisposition and development

In vitro normal bile duct cells express both IL-6 receptor and gp130 messenger RNA and protein, but their expression level is further increased in cholangiocarcinoma cells. Appearance of autocrine IL-6 production and high level of its receptors lead to autocrine/paracrine loop of IL-6 signaling during cholangiocarcinoma progression[5,109].

IL-6 target genes in cholangiocarcinoma progression

IL-6 enhances cell survival and proliferation of human malignant cholangiocytes by up-regulation of myeloid cell leukemia-1 (mcl-1) mRNA and protein expression which is a key member of the antiapoptotic Bcl-2 protein family[107,110]. Disruption of IL-6 signaling at different levels diminishes Mcl-1 promoter activity, its mRNA and protein level in human cholangiocarcinoma cell lines.

Sustained IL-6/JAK-STAT signaling and enhanced Mcl-1 expressions in cholangiocarcinoma are associated with at least partial epigenetic SOCS3 silencing. In human cholangiocarcinoma tissues and cell lines unusual inverse correlation exists between phosphorylated STAT-3 and SOCS3 protein expression. SOCS3 negative cholangiocarcinoma (as well as cholangiocarcinoma lines) samples are characterized by extensive methylation of SOCS3 promoter as compared with nontumor tissue. Treatment with demethylating agent restores IL-6 induction of SOCS3 leading to termination of phosphorylated STAT3 response, reduction of cholangiocarcinoma cells level of Mcl-1, and sensitization of cholangiocarcinoma cells to Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL)-mediated apoptosis[111,112].

GROWTH FACTOR INDUCTION OF STAT SIGNALING AND CHOLANGIOCARCINOMA DEVELOPMENT

HGF induced STAT signaling

HGF is a growth factor acting *via* receptor tyrosine kinase c-Met and inducing matrix dissociation, motility of epithelial cells, and enhancing invasiveness of tumor cells. In the 5'-flanking region of human HGF gene cis-acting STAT element has been found. Interruption of STAT3 signaling by dominant-negative STAT3 reduces HGF promoter activity[113,114].

Interaction of HGF with its receptor c-Met leads to receptor dimerization and phosphorylation of intracellular receptor domain which forms the docking sites for interaction with multiple downstream signaling molecules including STAT3. STAT3 activation has been shown to be necessary for full biological responses of HGF and HGF induced proliferation[115-117]. In hepatocytes and some other cell types HGF activates the STAT3 signaling pathway, moreover, not only STAT3 but also STAT1α/STAT1β and STAT5 interact with c-Met[118,119]. STAT3 phosphorylation induced by HGF/c-Met signaling is limited by consequent positive HGF action on SOCS3 expression in some cell types. SOCS3 has been observed to act as a negative regulator of HGF-induced cell migration[120-122].

Alterations of local and systemic HGF level

In situ hybridization studies have demonstrated that there is a close paracrine ligand-receptor relationship between HGF expressed in mesenchymal cells and c-Met expressed in the adjacent epithelial cells. HGF production is stimulated by various cytokines including IL-6-type cytokines. Both HGF and c-met mRNAs and protein products are overexpressed in cholestatic animal liver and human hepatolithiasis specimens[123-125]. Serum HGF level is elevated in cholangiocarcinoma patients[126]. Cholangiocarcinoma cell lines do not secrete HGF, but

their co-cultivation with neutrophils induces a high level of HGF secretion by these mesenchymal cells and may enhance invasiveness of tumor cells[127].

Alterations of HGF signaling

c-Met is expressed in cultured mouse biliary epithelial cells and liver bile ducts[123,128]. c-Met activation and elevation of c-met mRNA and protein expression have been shown in mouse chemically induced cholangiocarcinoma, different cholangiocarcinoma cell lines, and human cholangiocarcinoma patients. c-Met overexpression is more prominent in induced cholangiocarcinoma in rats and human cholangiocarcinoma cell lines as compared to normal and hyperplastic intrahepatic biliary epithelium[3,5,126,129-131]. The presence of HGF-c-Met-STAT3 positive autocrine/paracrine loop in cholangiocarcinoma may confer increased survival, growth, and invasiveness during cholangiocarcinoma progression. The malignancy of cholangiocarcinoma cells containing activated STAT3 may be at least partly due to stimulation of HGF expression in adjacent mesenchymal cells and c-met overexpression in tumor cells.

ERbB2 induced JAK-STAT and STAT signaling

ErbB2 also known as HER-2 or HER-2/neu is a member of ErbB receptor tyrosine kinase family acting mainly as the most common partner for heteromerization for other members of this receptor family (ErbB1, ErbB3 and ERbB2) among which EGF is the main ligand for ErbB1 (EGFR). ErbB receptors may be constitutively associated with JAK kinases. Direct association between ErbB2 and other ErbB receptors with STAT3 has also been found. Src kinases are the other molecules for ErbB receptor mediated STAT signaling and potential upstream regulators of JAK kinases. Ligand induced receptor dimerization leads to recruitment of Src kinase that induces JAK and STAT3 phosphorylation, dimerization and nuclear translocation[132,133]. Neuregulin induced heterodimers of ErbB2 and ErbB4 may activate STAT5[23]. Expression of SOCS1, 3, 4, 5 is elevated by EGF treatment[134-136]. Tumor cell types that over express ErbB2 preferentially activate prosurvival STAT signaling. In different tumors an autocrine loop between cytokines and ErbB receptors induces constitutive STAT3 activation[133,137].

In human non-neoplastic hepatic tissue ErbB2 mRNA and protein products have been found only in large mature bile ducts or have not been revealed at all[138-140]. Human gallstone disease and common bile duct ligation in rats are accompanied by appearance or elevation of ErbB2 expression in cholangiocytes as compared with normal liver but its expression was lower than in cases of cholangiocarcinoma[138,141,142]. ErbB2 overexpression has been revealed in different human biliary tract cancer cell lines, xenobiotic induced rodent cholangiocarcinoma, as well as in human cholangiocarcinoma tissue samples. Overexpression of both ErbB1 (EGFR) and ErbB2 may serve as a base for enhancement of EGF signaling in cholangiocarcinoma. Altered ERbB2 expression occurs early in cholangiocarcinogenesis and may play an important role in its progression[129,130,139,143,144].

PERSPECTIVE THERAPEUTIC ADDRESSING OF THE JAK-STAT PATHWAY

As illustrated above, signaling of most hormones, cytokines and growth factors involved in cholangiocyte regulation seems to be enhanced under the progression of cholangiocarcinoma. It is associated, not only with increment of their systemic levels, but also (probably mainly) due to their local hepatic secretion and increment of the expression of their receptors. Taking together these observations we can suggest that local manipulation of JAK-STAT activation can be a very perspective therapeutic approach for managing of cholangiocarcinoma at different stages of its progression. Recent advances in development of hormonal and cytokine antagonists (recombinant proteins for Prl and GH and peptide for IL-6[145-147]) give us an efficient tool for this treatment. Since synthetic specific inhibitors of JAK and other tyrosine kinases are intensively developed, we could suggest their efficient application for managing of cholangiocarinoma in addition to antagonists.

For the moment, the most specific and efficient way to inhibit the components of the JAK-STAT pathway is local expression (tissue-specific) of dominant negative analogues of JAKs and STATs. Evidently, their application is now limited to laboratory investigations and needs further development of genetic therapy. Nevertheless, it is a potent tool for precise pre-clinical investigations of JAK-STAT inhibition in cholangiocarcinoma. Thus, in the next chapter we focus on a detailed description of the main players of the JAK-STAT pathway in cholangiocarcinoma.

JAK-STAT SIGNALING UNDER CONDITIONS OF CHOLANGIOCARCINOMA PREDISPOSITION AND DEVELOPMENT

Presented data indicate that a number of receptors associated with JAK-STAT signaling are expressed in normal cholangiocytes, their expression increases under conditions of cholangiocarcinoma predisposition and is additionally elevated in cholangiocarcinoma cells (Table 1). Preferably used components of JAK-STAT signaling for such receptors are shown in Table 2. Thus, STAT5 and STAT3 proteins are the main downstream components for hormones, growth factors, and cytokines using JAK-STAT signaling in normal, proliferating, and malignant cholangiocytes and both STAT3 and STAT5 are implicated in promotion of cell survival in a variety of other normal and cancer tissues. SOCS3 is the primary negative regulator of cholangiocyte JAK-STAT signaling (Table 2). Among all these components, only STAT3 and SOCS3 have been investigated in cholangiocarcinoma cells.

STAT3

STAT3 plays an important role in transduction of survival signals downstream of both JAKs and receptor tyrosine kinases. Aberrant and frequently constitutive activation of STAT3 has been described in a variety of human cancers. Activation of STAT3 in tumors is associated also with tumor escape from immune attack[151,152].

Table 1 Cholangiocyte receptors associated with the JAK-STAT signaling pathways: alterations of expression during cholangiocarcinoma predisposition and development

Receptor type	Norma	Direction of alteration		References
		Cholangiocarcinoma predisposition	Cholangiocarcinoma development	
Prolactin receptor	Low	Up	Up Up	[7,58,148]
IL-6 receptor/gp130	Present	Up	Up Up	[2,5]
ErbB-2	Low/Absent	Up	Up Up	[2,138-140]
c-Met	Present	Up	Up Up	[2,5]
Growth hormone receptor	Moderate	Up	?	[72]
Leptin receptor	Present	?	?	[85]

Table 2 Known components of JAK-STAT signaling for receptors expressed in cholangiocytes

Receptor type	JAK	STATs	Negative regulators of JAK-STAT signaling	References
Prolactin receptor	JAK2	STAT5 > STAT3 > STAT1	SOCS3, SOCS1, SOCS2, CIS	[47,48,51-53]
Growth hormone receptor	JAK2	STAT5 > STAT3 > STAT1	SOCS3, SOCS1, SOCS2, CIS	[70,71]
Leptin receptor	JAK2	STAT3 > STAT5, STAT6, STAT1	SOCS3	[74-79]
IL-6 receptor/gp130	JAK1, 2, Tyk2	STAT3 > STAT1	SOCS3	[15,18-20,101,102,110-112]
ErbB-2	No, JAK3, TYK2	STAT3 > STAT5, STAT6	SOCS3, SOCS1, SOCS4, SOCS5	[23,132-136,149,150]
c-Met	No	STAT3 > STAT5, STAT1	SOCS3	[115-122]

In normal liver phosphorylated STAT3 localizes in epithelial cells lining large bile ducts and peribiliary glands[104]. STAT3 was constitutively phosphorylated in tested cholangiocarcinoma cell lines but not in nonmalignant cholangiocytes. Mcl-1 promotor has STAT3 binding site. STAT3 site-directed mutagenesis decreases Mcl-1 promotor activity[111].

SOCS3

STATs activation is negatively regulated by SOCS proteins. SOCSs are generally considered as a kind of tumor suppressor gene. In some hepatocarcinoma cell lines aberrant methylation in STAT-binding sites of the SOCS3 gene has been associated with loss of SOCS3 function and enhancement of tumor cell growth and migration[153]. Epigenetic inactivation of tumor suppressor genes by hypermethylation is relevant to cholangiocarcinoma development[154]. Known sustained STAT3 activation during cholangiocarcinoma progression may be partly due to SOCS3 epigenetic silencing due to hypermethylation of its promoter in human cholangiocarcinoma tissues and cell lines. Inverse correlation has been observed between phosphorylated STAT3 and SOCS3 protein expression in cholangiocarcinoma specimens. In cholangiocarcinoma samples failing to express SOCS3, extensive methylation of SOCS3 promoter has been shown in comparison with paired nontumor tissue. Methylation of SOCS3 promoter is also identified in two cholangiocarcinoma cell lines. Treatment with demethylating agent leads to restoration of SOCS3 expression, subsequent termination of STAT activation, and reduction of cellular levels of Mcl-1[112].

Cholangiocarcinoma markers and STAT-responsible genes

Based on the data on molecular alterations in human cholangiocarcinoma reviewed by Sirica, 2005, we have tried to analyze whether cholangiocarcinoma marker genes have putative STAT response elements in their promoters. As shown in Table 3 a number of genes involved in cholangiocarcinoma growth signaling, cell cycle regulation, and apoptosis inhibition possess candidate STAT3-, STAT5-, and STAT-1-binding sites in their promoters. Prl and IL-6 induced STAT5 and STAT3 activation is essential for up-regulation of their transcription activity in different tumor types and leads mainly to tumor progression[2,16,86,107,110-112,155,156].

We can assume that cholangiocarcinoma markers (presented in Table 3) including growth factors, members of pro-inflammatory signaling pathway, CDK inhibitors, cyclins, members of the antiapoptotic Bcl-2 protein family including Mcl-1, Bcl-2, Bcl-XL, may serve as final potential molecular targets of JAK-STAT cholangiocarcinoma signaling induced by Prl, IL-6, HGF, EGF and some other molecules acting *via* JAK-STAT signaling. These promoters STAT-binding sites may be involved in overexpression of these markers in cholangiocarcinoma cells.

It is important to note that down-regulation of CDK inhibitors ($p21^{waf1/cip1}$, $p27^{kip1}$) is a characteristic feature of cholangiocarcinoma development while activated STATs up-regulate them in some other tissues (Table 3), thus the role of JAK-STAT signaling cannot be restricted to progression cholangiocarcinoma.

PRIMARY NUCLEAR JAK-STAT SIGNALING AND CHOLANGIOCARCINOMA DEVELOPMENT

Internalization pathways have also been demonstrated for signaling molecules associated with the JAK-STAT pathway. Nuclear accumulation of such molecules, ligand dependent nuclear translocation of corresponding receptors, nuclear localization of JAKs, and primary nuclear activation of STAT proteins have been revealed in different cell types and experimental systems. While

Table 3 Cholangiocarcinoma marker genes with promoter putative STAT responsible elements

Cholangiocarcinoma altered genes[1]	Direction of alteration[1]	Putative STAT responsible elements	Regulation by STAT	References
HGF	Up	STAT3	Up	[113,114]
VEGF	Up	STAT3	Up	[157]
COX-2	Up	STAT3, STAT5	Up	[158]
Cyclin D1	Up	STAT5	Up	[16,155]
p53	Down and up	STAT1	Up	[159]
$p21^{waf1/cip1}$	Down	STAT1, STAT3, STAT5	Up	[155,160,161]
$p27^{kip1}$	Down	STAT3	Up	[162]
Bcl-2	Up	STAT3	Up	[16]
Bcl-Xl	Up	STAT3, STAT5	Up	[155,163]
Mcl-1	Up	STAT3	Up	[111,112]
MUC1	Up	STAT3, STAT1	Up	[164]
MUC4	Up	STAT	Up	[165]

[1]Altered genes and direction of changes of their expression are adopted from[2].

membrane JAK-STAT signaling is the main signaling pathway in normal conditions, the importance of the primary nuclear pathway of JAK-STAT signaling rises under conditions of cell proliferation including preneoplastic and neoplastic proliferation. Appearance of components of JAK-STAT signaling in the nucleus or the enrichment of their nuclear pool seems to be associated with their participation in the regulation of proliferative processes and may be related to cholangiocarcinoma progression.

Ligands (such as Prl, GH, leptin, IL-1, IL-5, IFN-γ, TGF-α) and receptors acting *via* the JAK-STAT pathway or associated with STAT signaling by other mechanisms have been found in the nuclear compartment of different cell types mainly under conditions of high proliferative status and cancer development[8,10,166-169].

Nuclear translocation of Prl bound to Prl receptors has been revealed after Prl stimulation of Nb2 Prl-dependent lymphoma cells and the human breast cancer cell line T47D. Another mechanism suggests that Prl enters the nucleus in complex with cyclophilin B. In the nucleus this complex interacts with STAT5 and potentiates its activity facilitating STAT5 DNA binding. Prl/cyclophilin B complex directly interacts with the STAT5 N-terminus and appears to induce dissociation of PIAS3 from its complex with STAT5[170,171].

Both GH and GH receptor are subject to rapid nuclear translocation. Rat growth hormone binding protein (GHBP) that is a product of alternative splicing of GH receptor gene with an intact extracellular domain and without transmembrane and intracellular domains replaced by short carboxyterminal sequences, is also localized in cell nuclei in *in vitro* and *in vivo* experiments upon GH stimulation. Nuclear localized GHBP acts as a potent enhancer of STAT5 mediated transcription presumably by binding to PIAS proteins and thereby enhances the action of both GH and other members of the cytokine receptor superfamily[9,172].

Receptor tyrosine kinases associated with the JAK-STAT pathway or STAT signaling have also been found in the nuclear compartment. Nuclear EGF receptor (ErbB1) has been detected in highly proliferative tissues, such as regenerating liver, specimens of primary tumor, and cancer cell lines. Expression of nuclear ErbB1 correlates positively with increased level of cyclin D1 and negatively with overall survival in breast cancer patients. Nuclear ErbB1 co-localizes and interacts with importins α1β1, carriers that are critical for macromolecule nuclear import[8,168,169,173-176]. ErbB1 might act as a transcription factor to activate genes required for high proliferating activity. After EGF stimulation and ErbB1 nuclear translocation EGF-ErbB1 complex associates with cyclin D1 promoter element designated AT-rich sequence (RTRS)[177].

The ability of nucleus translocation is also inherent for other receptors of the ErbB family (ErbB2, ErbB3, and ErbB4). Rat p185neu, ErbB2, and ErbB3 receptors exist in the nucleus as full length molecules, while ErbB4 may undergo γ-secretase-mediated cleavage and with nuclear translocation of C-terminal 80-kDa fragment that represents a soluble cytoplasmic domain with an active tyrosine kinase region[10,178]. Nuclear ErbB2 associates with a specific nucleotide sequence (named HER-2-associated sequence - HAS) of the COX-2 gene promoter and is able to activate its transcription[168,173,175-178].

The hypothesis of a primary nuclear JAK-STAT signaling pathway is supported by data on constitutive expression of JAK1 and JAK2 in the cell nucleus. JAK1 and JAK2 molecules have been shown to contain nuclear localization sequences and both kinases have been revealed in nuclei of rat hepatocytes, pancreatic islet cells, CHO and some other cell types both without activation by upstream signaling and in active phosphorylated forms. GH treatment of cells stimulates phosphorylation of nuclear JAK2 without apparent changes of its subcellular localization. Active nuclear JAK2 phosphorylates transcription factor - nuclear factor 1-C2 (NF1-C2) in mammary epithelial cells[8,179,180]. Since both JAK1 and JAK2 are expressed constitutively in the nucleus and a number of cytokine receptors undergo nuclear translocation the realization of primary nuclear JAK-STAT signaling pathway seems very possible.

Primary nuclear STAT activation has also been observed. Nuclear ErbB1 has been shown recently to interact with STAT3 in the nucleus leading to transcriptional activation of inducible iNOS gene in breast carcinoma cells and this pathway may be associated with a high malignancy of other types of tumor cells with high level of nuclear ErbB1 and

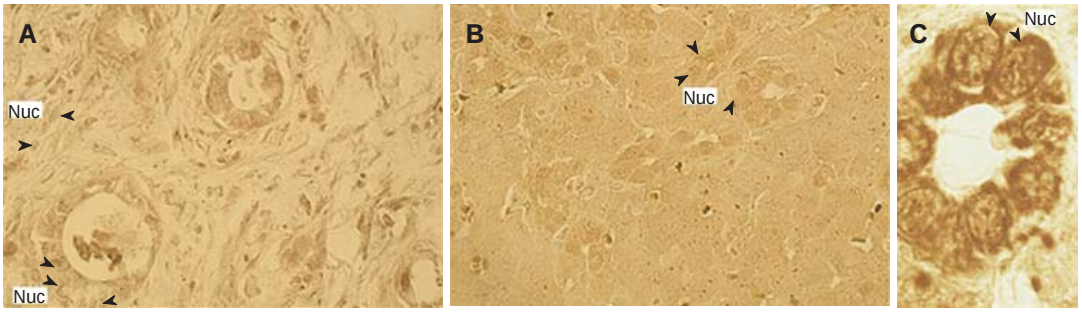

Figure 3 Immunohistochemistry for prolactin receptor (PrlR) expression in human cholangiocarcinoma sample (**A**) and in female (**B**) and male (**C**) rat liver tissue 14 d after common bile duct ligation. Intensive nuclear (Nuc), PrlR-positive immunoreactivity is shown. Orig. mag, **A**, **B**: x 40; **C:** x 100.

Table 4 Nuclear prolactin receptor expression in rat liver transplanted RS-1 cholangiocarcinoma cells and adjacent hepatocytes as compared with normal cells

	Nuclear PrlR-positive immunoreactivity (arbitrary units), medians (upper and lower quartiles)			
	Normal cholangiocytes	RS-1 cholangiocarcinoma cells	Normal hepatocytes	Tumor adjacent hepatocytes
Males	47 (0; 104)	191[b] (119; 288)	40 (26; 90)	308[b] (227; 381)
Females	113 (47; 194)	408[b] (256; 555)	59 (41; 101)	415[b] (266; 581)

[b]$P < 0.001$ as compared with correspondent intact group, Mann-Witney *U*-test.

STAT3[10]. C-terminal 80-kDa fragment of ErbB4 with nuclear traffic activity may associate with STAT5a in the nucleus and transactivate gene expression[178,181]. Intracellular ErbB4 domain possesses a nuclear localization sequence essential for its nuclear accumulation. In cotransfection experiments nuclear translocation of intracellular ErbB4 domain has been shown to be required for nuclear translocation of STAT5a and ErbB4 function as a nuclear chaperone for STAT5a transcription factor as has been suggested[182].

Thus, primary nuclear JAK-STAT signaling may become important at certain stages of the cell cycle or be critical for some populations of rapidly dividing or neoplastic cells.

Is a direct nuclear JAK-STAT pathway related to cholangiocarcinoma development? Intensive nuclear Prl receptor accumulation has been revealed in our laboratory in rat bile duct cells only after normal cholangiocyte transition to active proliferation during postnatal ontogenesis or after common bile duct ligation and is accompanied by elevation of total receptor content both in males and females[7]. Similarly, we have found nuclear localized Prl receptors with sharp elevation of their expression in samples of human cholangiocarcinoma tissues (Figure 3) as well as in liver transplanted rat cholangiocarcinoma cell line RS-1.

Both total content and nuclear expression of Prl receptors in transplanted RS-1 rat cholangiocarcinoma cells show moderate sex dependence with female predominance (Table 4). The intrahepatic RS1 cholangiocarcinoma transplant induces nuclear accumulation of Prl receptors not only in malignant cholangiocytes but also in adjacent and distant hepatocytes and increases total Prl receptor immunoreactivity in these cells[7,58].

We were unable to reveal prominent nuclear Prl receptor expression in hepatoma H27 cells after intrahepatic transplantation. Thus, nuclear accumulation of Prl receptors as well as their overexpression in intrahepatically transplanted RS1 cholangiocarcinoma cells and in hepatocytes of tumor bearing animals can serve as a specific marker of cholangiocarcinoma. Since we have used monoclonal antibodies U5 to the extracellular domain of rat Prl receptor, it was revealed that nuclear accumulation may be due to both full length Prl receptor or Prl binding protein as have been found for GH binding protein[9,172]. Appearance of Prl receptors in the nucleus is associated with Prl participation in the regulation of the proliferation process. This is confirmed by the data indicating that in Prl-dependent Nb2 lymphoma cells the cell cycle progression stops at the early G1 phase in the absence of Prl. Treatment of such cells with Prl leads to its transport to the nucleus followed by cell cycle progression[8,183].

In this respect unusual nuclear localization of prostaglandin E2 receptor EP1 that belongs to the G-protein coupled receptor superfamily detected in human cholangiocarcinoma cell lines seems very interesting[109].

Presented data show that primary nuclear JAK-STAT signaling may be relevant to cholangiocarcinoma progression and unusual nuclear localization of components of the JAK-STAT cascade may be considered as a predisposition to cholangiocarcinoma development.

CONCLUSION

While the role of GH, Prl and interleukins in normal cholangiocytes and pathophysiological states has been studied during the last decade, to date, the role of the JAK-STAT pathway in cholangiocarcinoma progression has not really been investigated and its importance is underestimated. Nevertheless, the summarized data provide a generalized view of the implication of the central cytokine signaling in tumor development of

epithelial bile duct cells. A more detailed study of JAK-STAT involvement in cholangiocyte proliferative diseases can open the door for newer therapeutic strategies serving a real alternative to surgery.

Moreover, an engagement of the new model of cancer (cholangiocarcinoma) to studies of JAK-STAT signaling can give us an exhaustive understanding of its role in cancer development at different stages, which is not yet completely clarified (promotive role in neoplasia development[33] and inhibitory role in metastasis progression[45]). Moreover, it can help us to decipher new mechanisms of activation of the components of the JAK-STAT pathway like receptor and/or ligand nuclear localization.

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GASTRIC CANCER

High prevalence of osteoporosis in patients with gastric adenocarcinoma following gastrectomy

Jung Sub Lim, Sang Bum Kim, Ho-Yoon Bang, Gi Jeong Cheon, Jong-Inn Lee

Jung Sub Lim, Department of Pediatrics, Korea Cancer Center Hospital, Nowon-gu, Seoul 139-706, Korea
Sang Bum Kim, Ho-Yoon Bang, Jong-Inn Lee, Department of Surgery, Korea Cancer Center Hospital, Nowon-gu, Seoul 139-706, Korea
Gi Jeong Cheon, Department of Nuclear Medicine, Korea Cancer Center Hospital, Nowon-gu, Seoul 139-706, Korea
Supported by the Korea Institute of Radiological and Medical Sciences Clinical Research Fund and Korea Science and Engineering Foundation
Correspondence to: Jong-Inn Lee, MD, Department of Surgery, Korea Cancer Center Hospital, Gongneung-dong 215, Nowon-gu, Seoul 139-706, Korea. jilee@kcch.re.kr
Telephone: +82-2-9701217 Fax: +82-2-9782005
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Abstract

AIM: To evaluate the prevalence and predictive factors of osteoporosis in patients with gastric adenocarcinoma after gastrectomy.

METHODS: The study included 133 patients diagnosed with gastric adenocarcinoma but who did not undergo prior diagnostic work-up for osteoporosis. Bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DXA) and vertebral deformity was assessed by plain X-rays. We evaluated the effects of age, sex, body mass index (BMI), anemia, back pain, vertebral deformity, tumor staging, reconstruction type, and past medical history to determine predictive factors of osteoporosis in these patients.

RESULTS: The prevalence of osteoporosis in the lumbar spine was 38.3% (male, 28.9%; female, 54.0%), and 15.0% in the femoral neck (male, 10.8%; female, 22.0%). The vertebral deformity rate was 46.6% (male, 43.4%; female, 52.0%). Age, BMI and hemoglobin correlated with BMD ($P < 0.01$). In males, anemia and age > 64 years were independent predictive factors of osteoporosis in multivariate analysis. In females, back pain was an independent factor for osteoporosis.

CONCLUSION: The results of this study revealed that prevalence of osteoporosis and vertebral bone deformity rate were high in gastric cancer patients, regardless of post-gastrectomy duration and operation type. Early diagnosis and a proper management plan must be established in these patients.

Key words: Gastric adenocarcinoma; Gastrectomy; Bone mineral density; Osteoporosis; Vertebral deformity; Anemia; Back pain

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INTRODUCTION

Gastric cancer is one of the most common cancers in the world. Although decreasing in prevalence, it is still the second most common cause of cancer death in the world. The incidence is still very high in East Asia[1]. Although the overall prognosis of gastric cancer is poor, the 5-year survival rate for those who receive operative treatment is 55.6% in Korea. Due to early diagnosis and aggressive surgical intervention, the survival rate is > 90% in cases of early gastric cancer in Korea[2]. However, many surviving patients suffer from the sequelae caused by the surgical procedure. Recently, a 63-year-old woman, who underwent gastrectomy for gastric malignancy, spontaneously developed multiple fractures. It was speculated that the synergistic effects of gastrectomy and other osteoporosis risk factors led to multiple fractures[3]. Weight loss, malnutrition, anemia and osteoporosis are common after gastrectomy[4]. Following gastric resection, reduced cortical and trabecular bone mass have been reported, however, the mechanism remains unclear[5,6].

The american Gastroenterological Association (AGA) has recommended dual-energy X-ray absorptiometry (DXA) in patients who are alive at least 10 years after gastrectomy[6], based on many reports of post-gastrectomy bone disease[7-20]. Most studies have dealt with patients with peptic ulcer disease, and those focusing on gastric cancer patients are rare. Recently, most gastrectomies have been done in patients with gastric malignancy. However, the age at operation and operation type might differ between peptic ulcer and gastric cancer. The duration after gastrectomy to onset of osteoporosis may also be different. Among the population aged > 50 years old, gastric cancer detection and operation have recently increased in Korea[2].

In this study, we evaluated the prevalence of osteoporosis and vertebral bone deformity rate in patients

with gastric adenocarcinoma after gastrectomy. We evaluated the impact of tumor staging, operation type, and other risk factors of osteoporosis. We also evaluated the association between clinical manifestations and osteoporosis for early diagnosis of osteoporosis in gastric cancer patients.

MATERIALS AND METHODS

One hundred and thirty-three patients with gastric cancer seen between January and December 2006 at the Korea Cancer Center Hospital were included in this study. The criteria for entry in this study were gastric cancer patients: (1) diagnosed with adenocarcinoma; (2) treated with curative gastrectomy and followed up without recurrence; (3) aged > 50 years; (4) never previously diagnosed with osteoporosis, and who had not taken any medication, such as estrogen, bisphosphonate, corticosteroid, and vitamin D; (5) with no other disease that affected bone mineral density (BMD) (like alcoholism, renal failure, chronic liver disease, and immobility); and (6) who could take care of themselves and had an average level of physical activity. Patients' characteristics are described in Table 1. Eighty-four enrolled patients were men and 55 were women, ranging in age from 50 to 80 years (median, 63.9 years).

The authors interviewed all patients closely regarding the known risk factors of osteoporosis, such as family history of osteoporosis, previous fracture, type 2 diabetes, and other chronic diseases and medication states. Anthropometric data (height, weight and BMI), operation record (tumor stage, gastrectomy and reconstruction type), and pathology report were also closely reviewed.

BMD of the lumbar spine (L1-4), and four sites in the proximal femur (femoral neck, Ward's triangle, greater trochanter, and total hip) were measured by DXA (GE Prodigy; Lunar Radiation, Madison, WI, USA). The results of BMD were expressed as absolute values (g/cm^2), T scores (compared with young adults) and Z scores (compared with age-matched controls) were according to Korean reference values provided by the GE-Lunar database. Osteoporosis was defined as a T score of < -2.5 and osteopenia as a T score of -1.0 to -2.5 SD[21]. Lateral radiographs of the thoraco-lumbar spine (T-L spine) were evaluated to assess vertebral bone deformity. Vertebral bone deformity was defined when Genant semi-quantitative grading was higher than grade 1[22]. Low BMI was defined as < 18.5 kg/m^2. Anemia was defined as hemoglobin < 11 g/dL in women and < 13 g/dL in men. High serum alkaline phosphatase (sALP) was defined as > 127 U/L (normal limit; < 126 U/L). Serum albumin, AST/ALT and blood urea nitrogen (BUN)/creatinine were used to rule out severe malnutrition, and liver and renal disease.

Statistical analysis

Numbers and values were expressed as means ± SD. Mean quantitative values were compared by Student's *t* test. Differences in proportions among the groups were analyzed by the χ^2 test. Variables relevant to osteoporosis were assessed by univariate analysis: age (< 64 *vs* ≥ 64 years); sex (male *vs* female); low BMI (< 18.5 kg/m^2); tumor stage; gastrectomy and reconstruction type (subtotal gastrectomy with Billroth Ⅰ and Billroth Ⅱ reconstruction *vs* total gastrectomy with Roux-en-Y reconstruction); serum ALP level (< 127 *vs* ≥ 127 U/L); hemoglobin level (male, < 13 *vs* ≥ 13 g/dL; female, < 11 *vs* ≥ 11 g/dL); family history of osteoporosis; previous fracture; type 2 diabetes; bone pain; and vertebral deformity. Variables significant by univariate analysis ($P < 0.05$) were included in a multivariate regression analysis. Data analysis was performed with SPSS version 13.0 (Chicago, IL, USA). $P < 0.05$ was considered as statistically significant.

RESULTS

DXA and vertebral deformity

BMD distribution was different between male and female patients; therefore, we listed BMD according to sex in Table 2. The Z score of L1-4 BMD was decreased -0.55 ± 1.55 in male and -0.72 ± 1.49 in female patients. However, the Z score of the femur neck was 0.44 ± 0.97 in male and 0.74 ± 1.14 in female patients. The L1-4 BMD Z score was significantly decreased compared with the femur neck BMD Z score ($P < 0.01$).

Fifty-one (38.3%) patients had osteoporosis and 42 (31.6%) had osteopenia in lumbar spine BMD, while 20 (15.0%) patients had osteoporosis and 42 (31.6%) had osteopenia in femur neck BMD (Figure 1). The osteoporosis rate was 28.9% in male and 54.0% in female patients in lumbar spine BMD. Osteoporosis rate was 10.8% in male and 22.0% in female patients in femur neck BMD. The BMI change after gastrectomy (-2.0 ± 2.4, male *vs* -2.9 ± 2.4 female), anemia rate (24% male *vs* 58% female), and TNM stage differed between male and female patients (Table 1). Vertebral bone deformity rate was 43.4% in male and 52.0% in female patients.

There was no difference in osteoporosis rate at the lumbar spine (39.7% *vs* 37.3%) and that of femur neck (17.2% *vs* 13.3%) between early and advanced gastric cancer. There was no difference in BMD and osteoporosis rate among patients who underwent partial gastrectomy with Billroth Ⅰ (40.4%) or Billroth Ⅱ reconstruction (38.9%), and total gastrectomy with Roux-en Y reconstruction (34.4%) (Figure 2).

Factors for osteoporosis in gastric adenocarcinoma

With the exception of sex, three variables were correlated with lumbar spine BMD. The correlation coefficients of age, BMI and hemoglobin were -0.391, 0.415 and 0.274, respectively ($P < 0.01$). In multiple regression analysis, BMI and hemoglobin level were correlated with BMD in male patients ($P < 0.05$). Age was not a significant factor ($P = 0.13$). On the contrary, BMI and age were significant factors in female patients ($P < 0.01$).

In univariate analysis, six variables were related to osteoporosis (Table 3). Of these factors, age > 64 years, female sex, BMI < 18.5 kg/m^2, anemia, back pain, and vertebral deformity were significant predictors of osteoporosis. Multivariate analysis disclosed three independent predictors after stratification by sex: serum hemoglobin level ≤ 13 *vs* > 13 g/dL (odds ratio, 4.887; 95% CI, 1.404-17.011; $P = 0.011$), age > 64 *vs* < 64 years

Table 1 Patient characteristics (mean ± SD)

Variables			All patients (n = 133)	Male (n = 83)	Female (n = 50)	P
Age (yr)			63.9 ± 7.0	64.3 ± 6.8	63.3 ± 7.4	NS
Duration after gastrectomy (yr)			2.7 ± 2.4	2.8 ± 2.6	2.5 ± 2.2	NS
BMI (kg/m^2)			21.5 ± 3.3	21.7 ± 3.1	21.2 ± 3.7	NS
BMI change after gastrectomy (kg/m^2)			–2.2 ± 2.4	–2.0 ± 2.4	–2.7 ± 2.3	< 0.05
AGC/EGC (n)			75/58	42/41	33/17	NS
TNM stage		1	76	56	20	< 0.05
		2	26	15	11	
		3	25	10	15	
		4	6	2	4	
Operation type	Subtotal gastrectomy	Billroth Ⅰ	47	30	17	NS
		Billroth Ⅱ	54	34	20	
	Total gastrectomy	Roux - en - Y	32	19	13	
Bone pain			61	39	22	NS
BMI < 18.5 (kg/m^2)			21	10	11	0.07
Family history of osteoporosis			10	6	4	NS
Type 2 diabetes			13	8	5	NS
Anemia			49	20	29	< 0.01
sALP > 127 U/L			18	12	6	NS
Past history of fracture			24	14	10	NS
Vertebral deformity			62	36	26	NS

AGC: Advanced gastric cancer; EGC: Early gastric cancer; Anemia, hemoglobin < 11 g/dL in females, < 13 g/dL in males.

Table 2 BMD of 133 patients with gastric adenocarcinoma patients according to sex (mean ± SD)

	Male			Female		
	BMD	T score	Z score	BMD	T score	Z score
L1-4	0.979 ± 0.203	-1.65 ± 1.63	-0.55 ± 1.55	0.835 ± 0.243	-2.31 ± 1.94	-0.72 ± 1.49
Femur - neck	0.837 ± 0.145	-0.89 ± 1.13	0.44 ± 0.97	0.765 ± 0.170	-1.11 ± 1.41	0.74 ± 1.14
Ward's	0.658 ± 0.151	-1.72 ± 1.17	0.04 ± 1.01	0.588 ± 0.181	-2.26 ± 1.40	0.13 ± 1.13
Trochanter	0.768 ± 0.154	-0.12 ± 1.40	0.65 ± 1.28	0.622 ± 0.139	-1.20 ± 1.24	-0.07 ± 0.96
Total hip	0.911 ± 0.181	-0.25 ± 1.45	0.79 ± 1.32	0.803 ± 0.174	-1.12 ± 1.44	0.37 ± 1.13

T scores (compared with young adults); Z scores (compared with age-matched controls).

(odds ratio, 3.923; 95% CI, 1.202-12.805; P = 0.018) in male patients, and back pain (odds ratio, 10.272; 95% CI, 2.297-45.942; P = 0.001) in female patients (Table 4).

DISCUSSION

In the present study, we examined BMD in gastric adenocarcinoma patients who underwent curative surgery and analyzed the factors that were related to osteoporosis. Hemoglobin level in men and back pain in women were independent risk factors of osteoporosis by multivariate analysis.

The overall prevalence of osteoporosis in gastric adenocarcinoma patients aged > 50 years was 39.6%. The osteoporosis rate of lumbar spine BMD was 29.8% in male and 54.5% in female patients. However, the osteoporosis rate of femur neck BMD was 11.9% in male and 26.3% in female patients. This difference might be explained by the fact that the Z score of lumbar spine BMD was significantly lower than that of femur neck BMD. It is consistent with other studies that trabecular bone was more affected than cortical bone after gastrectomy[7,8]. In other DXA-based studies in post-gastrectomy patients, the prevalence of spinal osteoporosis was 22%-37% and the prevalence of femoral neck osteoporosis was 10%-61%[9-11]. In the present study, 24 patients (18.0%) reported osteoporotic fracture with severe pain, such as Colles', ankle and severe vertebral fractures. Sixty-one (45.9%) patients complained of back pain and 62 (46.6%) showed vertebral bone deformity. Thirty-three (24.8%) patients complained of persistent back pain with vertebral deformity. Although it is not clear whether vertebral deformity with back pain directly indicates vertebral fracture, Zittel *et al* have reported that the overall vertebral fracture rate after gastrectomy was 31% by CT[12]. Recently, Gallacher *et al* have reported that vertebral deformity was found in 47% of osteoporosis patients and 23% of normal subjects[23]. In our study, we found vertebral deformity in 64.7% of osteoporosis patients and 22.5% of those with normal BMD.

The prevalence of osteoporosis was high in females, as previously reported[5,11,13]. However, this was mainly due to the effect of sex[24]. When we compared the Z scores of each sex, there was no difference in the decreased proportion of BMD, as in previous reports[14]. With the same age, women showed a greater osteoporosis rate than men. Except for estrogen deficiency, a high rate of anemia and low BMI might play some role in osteoporosis. Tovey

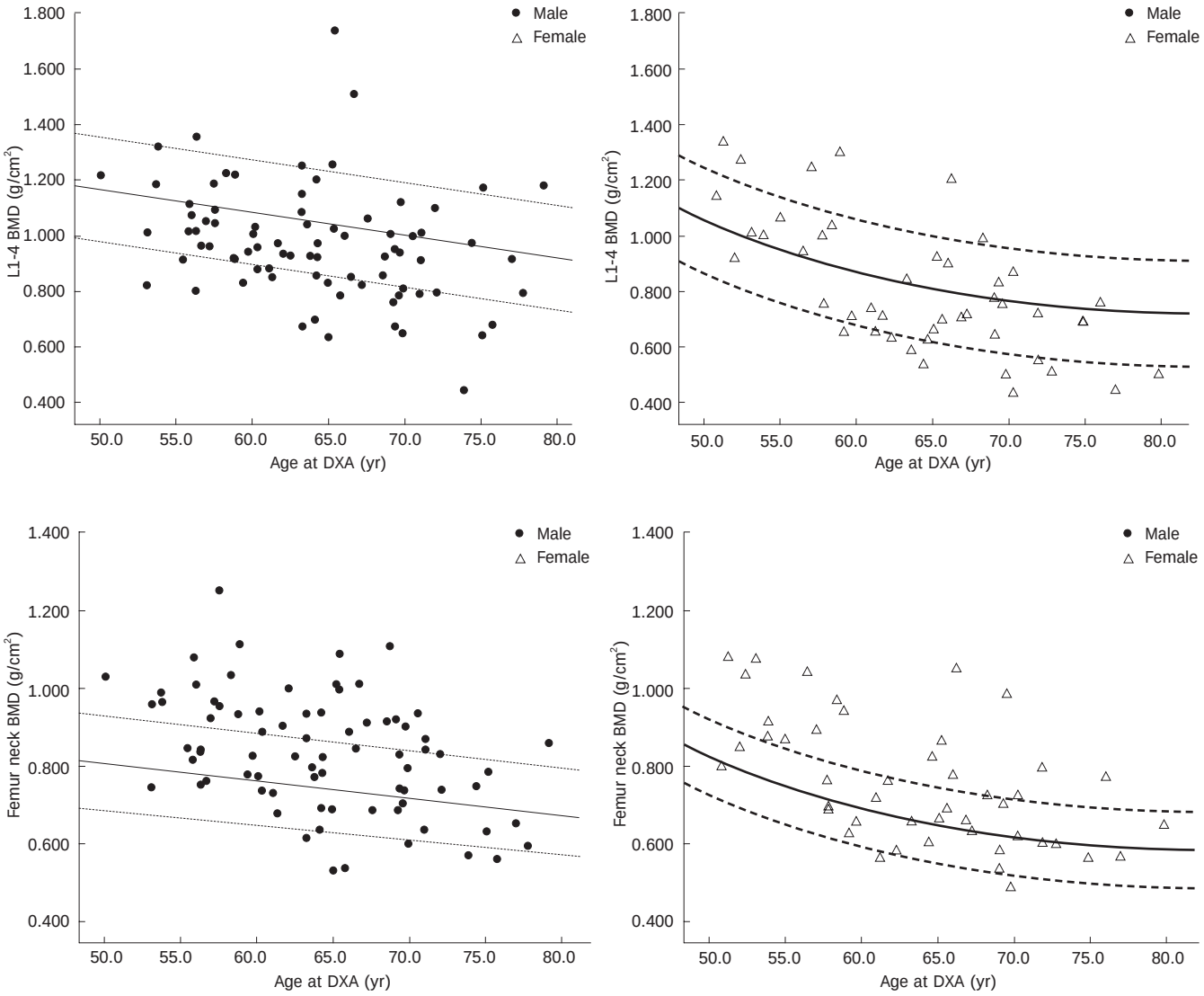

Figure 1 BMD of lumbar spine and femur neck. Solid line represents Z score = 0. Dashed line represents Z score = -1.0.

et al have also reported that iron deficiency anemia and osteoporosis are higher in female than in male patients throughout follow up after gastrectomy[5].

In the present study, there was no difference between BMD or osteoporosis rates between early and advanced gastric cancer. There was no difference in osteoporosis rates according to TNM stage. In addition, there was no difference in BMD and osteoporosis rate among patients undergoing Billroth Ⅰ, Billroth Ⅱ and Roux-en-Y reconstruction. Although some studies have shown that Billroth Ⅱ reconstruction patients have lower BMD compared to those undergoing Billroth Ⅰ reconstruction[15,16], our results showed no difference in osteoporosis rate in relation to surgical reconstruction type, as in previous studies[12,17]. These results indicate that gastrectomy itself affects BMD rather than the reconstruction method. The osteoporosis rate in gastrectomy patients cannot be predicted by reconstruction type, at least in surviving patients.

Anemia was a significant predictor for osteoporosis, at least in male patients. In multiple regression analysis, hemoglobin level was correlated with BMD in male patients ($P < 0.05$). In multivariate analysis, anemia was an independent predictor of osteoporosis (odds ratio, 4.887; $P = 0.011$). After gastrectomy and reconstruction, gastrointestinal physiology might be altered, and some nutrients might bypass the normal intestinal surface[6]. The main sites affected after gastrectomy are the duodenum and proximal jejunum, which are the main sites of calcium and iron absorption. In this study, we could not find an association between anemia and osteoporosis in females, as the rates were too high. The other factors such as age and BMI played a more important role in female patients. In females, 19 (86.4%) out of 22 patients who complained of back pain had osteoporosis. It is well known that new vertebral fractures are associated with substantial increases in back pain and functional limitation in older women[25].

This study had some limitations. We excluded patients who had been diagnosed as having previous osteoporosis. Therefore, selection bias might exist in many older patients and those who survived for long periods after gastrectomy. Although the postoperative duration or change in BMI did not show any impact on BMD or osteoporosis rate in our study, as in previous studies[17,18], some have reported

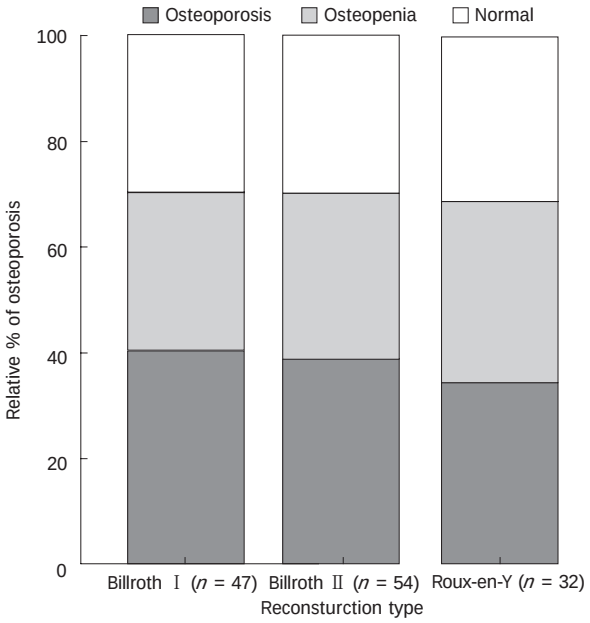

Figure 2 There was no difference in relative percentage of osteoporosis, osteopenia and normal BMD according to type of reconstruction in post-gastrectomy patients.

a negative correlation between BMD and the time interval after gastrectomy[14,19]. The other limitation was that 18 (13.5%) patients showed increased sALP ⩾ 127 U/L. In these cases, we could not rule out osteomalacia. Others have reported that 10%-20% of patients after gastrectomy might have osteomalacia[20,26].

In summary, we demonstrated high prevalence of osteoporosis and vertebral bone deformity in gastric cancer patients who underwent gastrectomy. Many also complained of back pain and a previous history of fractures. Considering the fact that gastric cancer patients after gastrectomy have many risk factors for osteoporosis fracture[27], early diagnosis and treatment are necessary. Anemia might be an important predictive factor of osteoporosis in the clinical setting. Although some early results suggest that post-gastrectomy osteoporosis is resistant to treatment[5], effective results have recently been reported with the development of many anti-osteoporosis drugs[28].

COMMENTS

Background

Post-gastrectomy bone disease may arise secondary to total or partial gastrectomy. The exact nature of the bone defect is unknown, although both osteoporosis and osteomalacia have been found. The incidence of osteoporosis is between 32% and 42%. These patients are associated with an increased risk of fracture. There is no difference in risk for post-gastrectomy bone disease between those undergoing a Billroth I and Ⅱ procedure.

Research frontiers

Recent studies have confirmed normal intestinal absorption of vitamin D in post-gastrectomy patients, although patients with post-gastrectomy steatorrhea have abnormal vitamin D absorption. Post-gastrectomy patients may alter their diet, and reduced serum 25-hydroxy-vitamin D level may in part reflect reduced dietary intake of vitamin D. Serum calcium and phosphate levels are most often normal post-gastrectomy, although calcium levels may be normal as a result of mobilization of calcium from bone. sALP, vitamin D metabolites and parathyroid hormone levels are variable post-gastrectomy. Patients who are at least 10 years post-gastrectomy (especially postmenopausal women, men aged > 50 years, and patients with low-trauma fractures), should undergo DXA testing.

Table 3 Univariate analysis for factors associated with osteoporosis

Variables	Patient number	*P*
Age (yr) < 64/⩾ 64	65/68	0.001
Male/female	83/50	0.006
Duration after gastrectomy (yr) < 3/⩾ 3	88/45	0.573
AGC/EGC	75/58	0.858
TNM stage Ⅰ/Ⅱ/Ⅲ/Ⅳ	76/26/25/6	0.164
Total/partial gastrectomy	31/102	0.528
Operation type Billroth Ⅰ/Ⅱ/Roux-en Y	47/54/32	0.858
BMI < 18.5/⩾ 18.5 kg/m^2	21/112	0.001
Anemia; males, Hb < 13/⩾ 13; females, Hb < 11/⩾ 11	49/84	0.001
sALP < 127/⩾ 127 U/L	115/18	0.040
Previous fracture history +/-	24/109	0.360
Family history of osteoporosis +/-	8/125	0.482
Type 2 diabetes +/-	13/111	0.369
Back pain +/-	61/72	0.001
Vertebral deformity +/-	62/71	0.001

Hb: Hemoglobin.

Table 4 Multivariate analysis for factors associated with osteoporosis after stratification by sex

	Variables	Odds ratio (95% CI)	*P*
Male	Anemia	4.887 (1.404-17.011)	0.011
	Age ⩾ 64 yr	3.923 (1.202-12.805)	0.018
Female	Back pain	10.272 (2.297-45.942)	0.001

Innovations and breakthroughs

This study confirms previous observations that the prevalence of osteoporosis is high in patients following gastrectomy. As noted previously, there is no difference in BMD and osteoporosis rate among patients undergoing Billroth Ⅰ, Billroth Ⅱ and Roux-en-Y reconstruction. A number of variables have been assessed in an attempt to identify factors that predispose to reduced BMD post-gastrectomy. The authors identified that hemoglobin level and age in men and back pain in women were independent risk factors of osteoporosis, by multivariate analysis. Increased time from surgery did not correlate with lower BMD.

Peer review

This is an interesting paper from South Korea. The data presented are clear and concise and have been described clearly in the text.

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GASTRIC CANCER

Effect of arsenic trioxide on vascular endothelial cell proliferation and expression of vascular endothelial growth factor receptors Flt-1 and KDR in gastric cancer in nude mice

Yan-Feng Xiao, De-Dong Wu, Shan-Xi Liu, Xi Chen, Li-Fen Ren

Yan-Feng Xiao, De-Dong Wu, Xi Chen, Li-Fen Ren, Department of Pediatrics, The Second Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710049, Shaanxi Province, China
Shan-Xi Liu, Department of Hematology, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710049, Shaanxi Province, China
Supported by The Science Fund of the Second Affiliated Hospital of the Medical College, No. 2003-YL-35
Correspondence to: Dr. Yan-Feng Xiao, Department of Pediatrics, The Second Affiliated Hospital of the Medical College, Xi'an Jiaotong University, Xi'an 710049, Shaanxi Province, China. xiaoyanfeng0639@sina.com
Telephone: +86-29-87679541 Fax: +86-29-88494931
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Abstract

AIM: To investigate the effect of arsenic trioxide (As_2O_3) on expression of vascular endothelial growth factor receptor-1 (VEGFR-1, Flt-1) and VEGFR-2 (KDR) in human gastric tumor cells and proliferation of vascular endothelial cells.

METHODS: The solid tumor model was formed in nude mice with the gastric cancer cell line SGC-7901. The animals were treated with As_2O_3. Microvessel density (MVD) and expression of Flt-1 and KDR were detected by immunofluorescence laser confocal microscopy. SGC-7901 cells were treated respectively by exogenous recombinant human $VEGF_{165}$ or $VEGF_{165}$ + As_2O_3. Cell viability was measured by MTT assay. Cell viability of ECV304 cells was measured by MTT assay, and cell cycle and apoptosis were analyzed using flow cytometry.

RESULTS: The tumor growth inhibition was 30.33% and 50.85%, respectively, in mice treated with As_2O_3 2.5 and 5 mg/kg. MVD was significantly lower in arsenic-treated mice than in the control group. The fluorescence intensity levels of Flt-1 and KDR were significantly less in the arsenic-treated mice than in the control group. $VEGF_{165}$ may accelerate growth of SGC7901 cells, but As_2O_3 may disturb the stimulating effect of $VEGF_{165}$. ECV304 cell growth was suppressed by 76.51%, 71.09% and 61.49% after 48 h treatment with As_2O_3 at 0.5, 2.5 and 5 μmol/L, respectively. Early apoptosis in the As_2O_3-treated mice was 2.88-5.1 times higher than that in the controls, and late apoptosis was 1.17-1.67 times higher than that in the controls.

CONCLUSION: Our results showed that As_2O_3 delays tumor growth, inhibits MVD, down-regulates Flt-1 and KDR expression, and disturbs the stimulating effect of $VEGF_{165}$ on the growth of SGC7901 cells. These results suggest that As_2O_3 might delay growth of gastric tumors through inhibiting the paracrine and autocrine pathways of VEGF/VEGFRs.

Key words: Arsenic trioxide; Gastric tumor; Flt-1; Tumor growth inhibition

Xiao YF, Wu DD, Liu SX, Chen X, Ren LF. Effect of arsenic trioxide on vascular endothelial cell proliferation and expression of vascular endothelial growth factor receptors Flt-1 and KDR in gastric cancer in nude mice. *World J Gastroenterol* 2007; 13(48): 6498-6505

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INTRODUCTION

Angiogenesis is recognized as playing a role in the pathophysiology of many human malignancies. It is an important factor in the progression and enlargement of solid neoplasms and is closely related to invasion and metastases[1-3]. Angiogenesis is influenced by a number of positive and negative regulatory factors, such as cytokines, extracellular matrix, and other cellular constituents including pericytes[4]. Among the factors contributing to angiogenesis, vascular endothelial growth factor (VEGF) is recognized as one of the most important molecules in the formation of new blood vessels. VEGF is a potent and specific mitogen for endothelial cells that activate the angiogenic switch *in vivo* and enhance vascular permeability. A variety of malignant human tumors are known to secrete VEGF, which has been correlated with the onset of angiogenesis in tumors[5-7]. Over-expression of VEGF is suggested to participate in the carcinogenic process[8]. VEGF binds to two distinct receptors on endothelial cells: VEGFR-1 (fms-like tyrosine kinase receptor 1, Flt-1) and VEGFR-2 (kinase insert domain containing receptor human homologue/fetal liver kinase 1

murine homologue, KDR/Flk-1)[9,10]. KDR is responsible for mitogenic signaling, and plays an important role in vasculogenesis and blood island formation, and Flt-1 regulates the assembly of endothelial cells and tissue factor production in endothelial cells[8]. Thus, it is suggested that inhibition of VEGF/VEGFR pathways may interrupt VEGF-induced angiogenesis. Recently, a few studies have demonstrated co-expression of VEGF and its receptors in tumor cells, which suggests that a VEGF autocrine pathway exists in tumor cells[11-14].

Arsenic is a common natural substance. Arsenic trioxide (As_2O_3) has shown substantial efficacy in treating both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL)[15]. Recent studies have shown that a wide variety of malignancies, including both hematological cancer and solid tumors derived from several tissue types, may be susceptible to therapy with As_2O_3[16-19]. These successes have prompted investigations to elucidate the mechanisms of action underlying these clinical responses. Emerging data suggest that arsenic induces apoptosis and inhibits tumor growth[15,16,20]. It has recently been reported that arsenic may inhibit angiogenesis[21-23]. We have recently shown *in vivo* and *in vitro* that As_2O_3 can inhibit VEGF expression and suppress angiogenesis and gastric tumor growth[24].

In the present work, we investigated further the effect of As_2O_3 on the expression of VEGF receptors Flt-1 and KDR in human gastric tumor cells and the proliferation of vascular endothelial cells. Our study demonstrated that As_2O_3 delayed tumor growth by inhibiting the paracrine and autocrine pathway of VEGF/VEGFRs.

MATERIALS AND METHODS

Animals and cells

Male Balb/c mice, 5-wk-old and weighing 19-21 g (from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were used in this study. The mice were kept in a laminar-filtered airflow cabinet under pathogen-free conditions with a constant temperature of 22℃ ± 2℃, relative humidity of 55% ± 5%, and 12-h dark/light cycles. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Fourth Military Medical University. Human gastric cancer cell line SGC-7901 and ECV304, a cell line derived from human umbilical vessel endothelial cells, were purchased from the Animal Laboratory Centre, Fourth Military University.

Tumor xenografts in nude mice

SGC-7901 cells were cultured in RPMI 1640 medium supplemented with 100 mL/L fetal bovine serum (FBS) at 37℃ in a 5 mL/L CO_2 incubator. Thirty mice received subcutaneous injection in the right flank with 200 μL cell suspension containing 2×10^7 SGC-7901 cells. After 10 d, when established tumors of 0.2 cm-0.3 cm diameter were detected, drug administration was started.

Drug treatment

The animals were randomly divided into three groups of 10 animals each. Arsenious acid [H_3AsO_3 ($As_2O_3 + 3H_2O_2 \Leftrightarrow H_3AsO_3$); Yida Pharmaceutical, Harbin, China] diluted with saline solution was injected intraperitoneally every day to the two treatment groups (2.5 and 5 mg/kg in 0.2 mL) and the same volume of saline solution was injected into the control group. After 10 d of treatment, three groups of mice were sacrificed and the tumor masses were removed. After the weight of tumor masses was measured, they were fixed in 40 g/L paraformaldehyde, and the frozen sections were prepared with a cryomicrotome (HM505E, Microm, Germany) for immunofluorescence analysis.

Tumor inhibition and regression

Tumor growth inhibition (TGI) was calculated by measuring tumor volumes before treatment, and 6 and 11 d after treatment. Tumor volume (cm^3) was calculated using the formula: tumor volume = $1/2ab$, where a is the long axis, and b the short axis. TGI (%) = $(1 - Vt/Vc) \times 100\%$, where Vt is the mean tumor volume of the arsenic-treated group, and Vc the mean tumor volume of the control group.

Microvessel density (MVD)

For identification of microvascular structure, frozen sections were cut and stained with rat anti-mouse CD31 (Biolegend, USA) and secondary antibody (goat anti-rat IgG conjugated TRITC). MVD was determined by confocal microscopy of tumor tissue sections. A single microvessel was defined as any immunofluorescent endothelial cell that was distinguished from adjacent tumor cells and other connective tissue elements. The microvessels were carefully counted in 20 fields (×400). The mean ± SE was expressed as the number of microvessels identified within the area.

Immunofluorescence for Flt-1 and KDR

The frozen sections were kept at room temperature for 30 min, incubated in distilled water and PBS for 5 min each, permeabilized in 1 g/L Triton-X-100 for 10 min, washed with PBS, blocked with 100 mL/L sheep serum (Sigma, USA) at 37℃ for 20 min, incubated with the primary antibody (Flt-1 and KDR rabbit polyclonal antibodies; Lab Vision, Fremont, CA, USA) at 4℃ overnight, washed with PBS, incubated with the secondary antibody (sheep anti-rabbit IgG conjugated FITC, diluted 1:100; Sigma) for 1h at 37℃, washed with PBS, and then examined by a TCS SP2 laser confocal microscope (Leica, Wetzlar, Germany).

For each group, several field images of Flt-1 or KDR were observed under confocal microscopy. The fluorescence intensity of each cell in the confocal fluorescence images was measured using the Leica Confocal analysis system, and the mean fluorescence intensity in a group of cells was then calculated.

Cell viability assay

ECV304 cells were seeded in a 96-well plate (2×10^3 cells/well). After 48 h seeding, cells were treated with As_2O_3 (0.5, 2.5 and 5 μmol/L) for 2 d, in three parallel wells each, and untreated cells served as a control. By 48 h, 20 μL

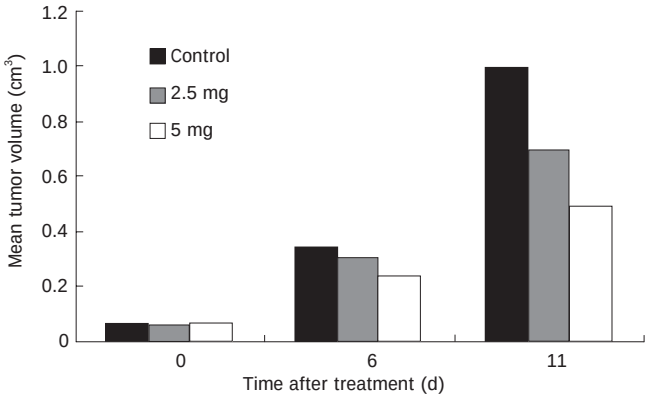

Figure 1 Arsenic trioxide inhibition gastric tumor xenografts growth.

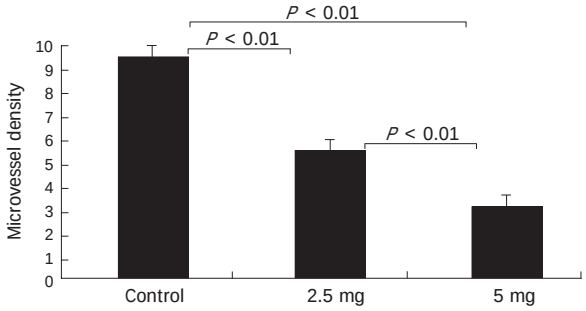

Figure 2 Effect of arsenic trioxide on tumor angiogenesis. Microvessel density (MVD) was measured with counting the fluorescence staining cell in high power field (400). Data are given as mean and SE.

MTT (15 mg/mL) was added to each well and incubated for a further 4 h. The medium was removed and 150 μL DMSO was added to each well. A_{492} was measured using a microculture reader. The percentage of viable cells was calculated as follows: (A of experimental group/A of control group) × 100%.

SGC-7901 cells were seeded in a 96-well plate (2×10^3 cells/well). After sedimentation, exogenous recombinant human $VEGF_{165}$ (2.5, 5, 15 and 20 ng/mL) or $VEGF_{165}$ (2.5, 5, 15 and 20 ng/mL) + 2.5 μmol/L As_2O_3 or $VEGF_{165}$ (2.5, 5, 15 and 20 ng/mL) + 5 μmol/L As_2O_3 were added to the medium, in three parallel wells each, and cultured for a further 48 h. For control wells, an equal volume of medium was added. The methods for the MTT assay and calculation of the percentage of viable cells were the same as above.

Flow cytometry by annexin V-FITC conjugated with propidium iodide (PI) staining

ECV304 cells were seeded in a 24-well plate (1×10^6 cells/well). After 48 h seeding, cells were treated with As_2O_3 (0.5, 2.5 and 5 μmol/L) for 2 d, and untreated cells served as controls. By 48 h, the cells were washed twice with cold PBS and then resuspended in a binding buffer at a concentration of 1×10^6 cells/mL, and the 100 μL solution (1×10^5 cells) was transferred to 5-mL culture tubes. Five microliters of annexin V-FITC and 10 μL PI (μg/mL) were added to each 100-μL solution, and the cells were gently vortexed and incubated for 15 min at room temperature in the dark. The samples, to which 400 μL PBS was added, were analyzed by FACScalibur flow cytometer (BD Biosciences, USA). Early apoptosis was estimated by the relative amount of $FITC^+PI^-$ cells.

Flow cytometry by PI staining

ECV304 cells were treated with As_2O_3 0.5, 2.5 or 5 μmol/L. By 48 h, the harvested cells were fixed with 1 mL of 750 mL/L cold ethanol at 4℃ overnight, and then washed with PBS. The cells were incubated with RNAse and PI for 30 min in the dark. Cell cycle of samples was analyzed using FACSCalibur flow cytometry.

Statistical analysis

The data were represented as mean ± SD. Data were analyzed with SPSS 10.0 statistical software (SPSS, Chicago, IL, USA). Multiple statistical comparisons were performed using ANOVA in a multivariate linear model. The Student-Newman-Kewls test was used to assess differences between the treatment and control group. $P < 0.05$ was considered statistically significant.

RESULTS

As_2O_3 inhibition of growth of human gastric tumor xenografts

All 30 nude mice developed tumors 10 d after implantation of SGC-7901 cells. When drug administration was started, there was no significant difference in the tumor volume of the three groups (control group, 67 ± 45 mm^3; 2.5 mg/kg As_2O_3 group, 63 ± 36 mm^3; 5 mg/kg group, 66 ± 48 mm^3, $P > 0.05$). Tumor volume in the three groups from 0, 6 and 11 d after treatment is shown in Figure 1. There were significant differences in the tumor volumes in the arsenic-treated groups (2.5 mg/kg, 696 ± 125 mm^3 and 5 mg/kg, 491 ± 116 mm^3) and control group (999 ± 338 mm^3, $P < 0.05$). On the other hand, the tumor volume in the 5 mg/kg As_2O_3 group was significantly less than that in the 2.5 mg/kg group ($P < 0.05$). TGI was 30.33% (2.5 mg/kg group) and 50.85% (5 mg/kg group) after arsenic treatment.

As_2O_3 inhibition of tumor angiogenesis

Sections of tumors were stained for CD31 immuno-fluorescence to detect the number of endothelial cells (ECs) as a measure of tumor angiogenesis. MVD was significantly lower in the 2.5 mg/kg As_2O_3 group (9.32 ± 0.33 *vs* 5.36 ± 0.32, $P < 0.01$) and 5 mg/kg group (9.32 ± 0.33 *vs* 3.05 ± 0.24, $P < 0.01$) than in the control group. MVD was significantly lower in the 5 mg/kg group than in the 2.5 mg/kg group. These result demonstrated the decreased capillary density of the tumor after treatment with As_2O_3 (Figure 2).

Effect of As_2O_3 on Flt-1 and KDR expression in tumor xenografts

Expression of Flt-1 and KDR was confirmed by the presence of fluorescence-stained cytoplasm in the tumor cells. Stronger immunoreactivity to Flt-1 (Figure 3A) and KDR (Figure 4A) was found in all the SGC-7901 tumor xenografts of the

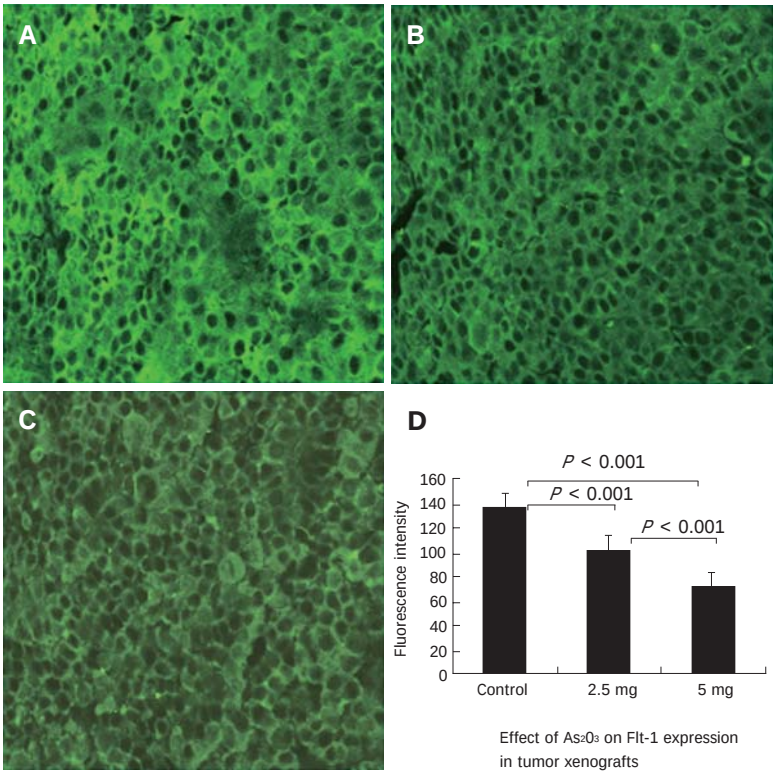

Figure 3 Effective inhibition of expression of Flt-1 (VEGFR-1) in the xenograft tumors by treatment with arsenic trioxide. Flt-1 expression was determined by Immunofluorescence staining and laser confocal microscope. Original magnification × 400. **A**: Control group; **B**: 2.5 mg/kg group; **C**: 5 mg/kg group; **D**: Quantitative analysis of fluorescence intensity in the three groups of tumors was measured using the Leica Confocal analysis system. Data are given as mean and SE.

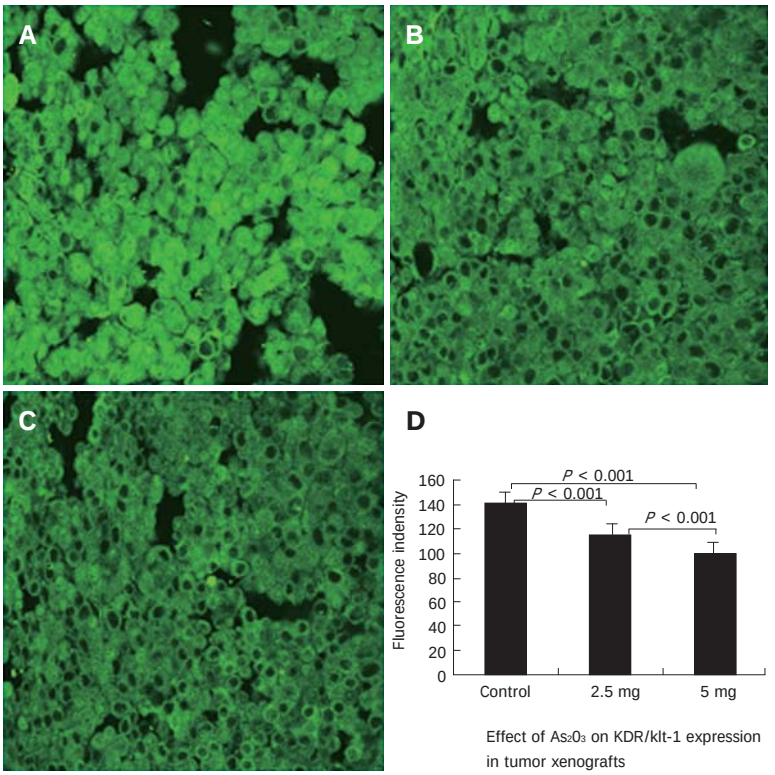

Figure 4 Effective inhibition of expression of KDR (VEGFR-2) in the xenograft tumors by treatment with arsenic trioxide. KDR expression was determined by Immunofluorescence staining and laser confocal microscopy. Original magnification × 400. **A**: Control group; **B**: 2.5 mg/kg group; **C**: 5 mg/kg group; **D**: Quantitative analysis of fluorescence intensity in the three groups of tumors was measured using the Leica Confocal analysis system. Data are given as mean and SE.

control group. The weaker fluorescence intensity was observed in tumor cells of the mice treated with 2.5 mg/kg (Figures 3 and 4B) and 5 mg/kg (Figures 3 and 4C) As_2O_3. The expression of Flt-1 and KDR in tumor cells was significantly less in the arsenic-treated groups than in the control group ($P < 0.001$). Expression of Flt-1 and KDR in the 5 mg/kg As_2O_3 group was less than that in the 2.5 mg/kg group (Table 1).

Effect of As_2O_3 on VEGF-stimulated growth of SGC7901 tumor cells

SGC7901 cells were incubated with varying concentrations of $VEGF_{165}$ or $VEGF_{165}$ + 2.5 μmol/L As_2O_3 and varying concentrations of $VEGF_{165}$ + 5 μmol/L As_2O_3. Its effects were measured using the MTT assay (Table 2). The results showed that $VEGF_{165}$ may have accelerated the growth of SGC7901 cells,

Table 1 Effect of As_2O_3 on Flt-1 and KDR/Flk-1 expression in tumor xenografts (fluorescence intensity) (n = 10, mean ± SD)

Group	Flt-1	KDR/Flk-1
Control	137.41 ± 3.36	141.62 ± 1.66
2.5 mg/kg	103.53 ± 2.23[b]	114.99 ± 2.19[b]
5 mg/kg	72.17 ± 0.87[b,d]	101.11 ± 1.89[b,d]

[b]P < 0.001, treatment groups *vs* control group; [d]P < 0.001, 5 mg/kg group *vs* 2.5 mg group.

Table 2 Effect of As_2O_3 on VEGF-stimulated growth of tumor cells (%)

	As_2O_3 (ng/mL)				
	2.5	5	10	15	20
$VEGF_{165}$	105.79	106.62	100.97	107.59	109.38
$VEGF_{165}$ + 2.5 μmol/L As_2O_3	84.24	97.95	97.59	100.48	94.94
$VEGF_{165}$ + 5 μmol/L As_2O_3	76.87	68.55	83.74	85.06	84.94

but As_2O_3 may have disturbed the stimulatory effect of $VEGF_{165}$.

Effect of As_2O_3 on proliferation of vascular endothelial cells

Cell viability was determined by the MTT assay (Figure 5). As_2O_3 inhibited the growth of ECV304 cells in a dose-dependent manner. Cell growth was suppressed by 76.51%, 71.09% and 61.49% after 48 h treatment with As_2O_3 at 0.5, 2.5 and 5 μmol/L, respectively.

Cell cycle and apoptosis of vascular endothelial cells induced by As_2O_3

Flow cytometry with only PI staining showed that the percentage of cells treated with As_2O_3 was higher in the sub-G1 period, lower in the sub-S period, and lower in the G2/G1 period when compared to the controls. Apoptotic cells were respectively 14.84% and 18.9% in the 2.5 and 5 μmol/L As_2O_3 groups (Table 3 and Figure 6). Early apoptotic cells, detected by flow cytometry with annexin V conjugated with PI staining, reached, respectively, 4.18, 5.36 and 7.4% in the 0.5, 2.5 and 5 μmol/L As_2O_3 groups (Figure 7). Early apoptosis in As_2O_3-treated groups was 2.88-5.1 times higher than that of the controls, and late apoptosis was 1.17-1.67 times than that of the controls.

DISCUSSION

Angiogenesis is critical for supporting the rapid growth of tumors. Angiogenesis inhibition is a promising therapeutic approach for the treatment of cancer. As_2O_3 has been demonstrated to induce complete remission in patients with APL without severe toxicity. More recently, *in vitro* studies have shown As_2O_3 apoptosis in other leukemia and solid tumor cells. As a novel anticancer agent, a few *in vivo* investigations of its efficacy on solid tumors have been carried out[19,21]. In this study, we observed the effect of As_2O_3 on growth and angiogenesis in a gastric cancer

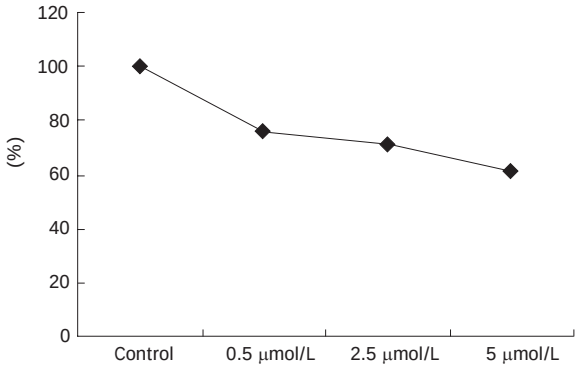

Figure 5 Effect of As_2O_3 on growth of ECV304 cells. The cell viability was analyzed by MTT assay.

Table 3 Effect of As_2O_3 on cell cycle of ECV304 cells (%)

Groups	G1	G2/G1	S	Apoptosis
Control	94.24	2.05	5.76	0
0.5 μmol/L As_2O_3	96.17	1.81	2.59	0
2.5 μmol/L As_2O_3	96.15	1.91	t0	14.84
5 μmol/L As_2O_3	96.96	1.84	0	18.90

SGC7901 xenograft model. MVD was measured in tumor tissue by the amount of labeled CD31. The results revealed that a 10 d treatment with As_2O_3 resulted in tumor growth inhibition and MVD decrease. TGI was respectively 29.08% and 52.17% in mice treated with 2.5 and 5 mg/kg As_2O_3. MVD was significantly lower in tumor tissues in arsenic-treated mice than in the control groups. The results demonstrated that As_2O_3 inhibited gastric cancer growth *in vivo* and suppressed the formation of new blood vessels in cancer tissues. Anti-angiogenesis may be one of the mechanisms by which As_2O_3 inhibits gastric cancer growth.

ECs are the primary structured units of blood vessels. In recent years, ECs have been the target cell of some angiogenesis inhibitors that have been undergoing clinical trials. These inhibitors can selectively affect diverse EC functions that are related to angiogenesis, including activation, proliferation, migration, invasion and survival[25,26]. It has been reported that particles of realgar (As_2S_2) with an average diameter of 100-150 nm can induce EVCV-304 cell apoptosis[27]. We studied the effect of As_2O_3 on growth and proliferation of ECV304 cells. In our study, the MTT assay showed that As_2O_3 inhibited the growth of ECV304 cells in a dose-dependent manner. Flow cytometry assays with only PI staining showed that the percentage of the cells treated with As_2O_3 were higher in the sub-G1 period, lower in the sub-S period, and lower in the G2/G1 period when compared to the controls; and 14.84% and 18.9% of the cells treated with As_2O_3 at 2.5 and 5 μmol/L, respectively, were apoptotic. Early apoptosis in the As_2O_3 treated groups was 2.88-5.1 times higher than that in the controls, and late apoptosis was 1.17-1.67 times higher than that in the controls. These results showed that As_2O_3 inhibited the viability of ECV304 cells and arrested the cells in G1 phase, blocked or delayed their entry into S phase, disturbed DNA synthesis, and induced apoptosis in

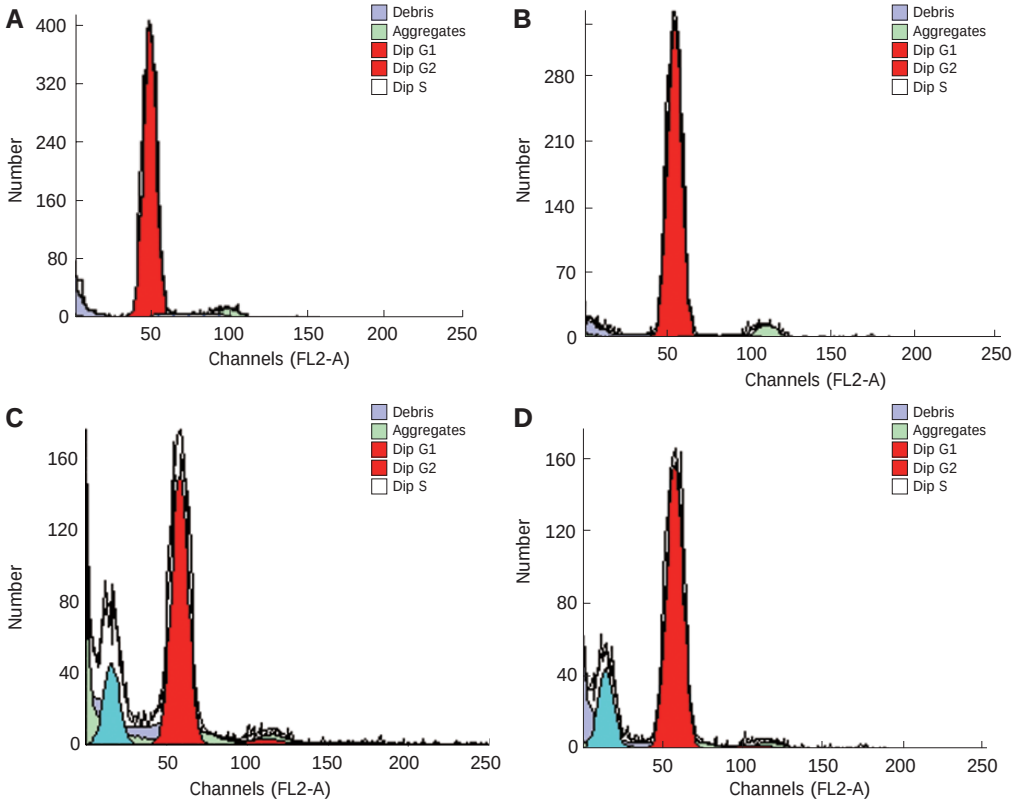

Figure 6 Effect of As_2O_3 on cell cycle of ECV304 cells. **A**: Control; **B**: 0.5 μmol/L As_2O_3; **C**: 2.5 μmol/L As_2O_3; **D**: 5 μmol/L As_2O_3.

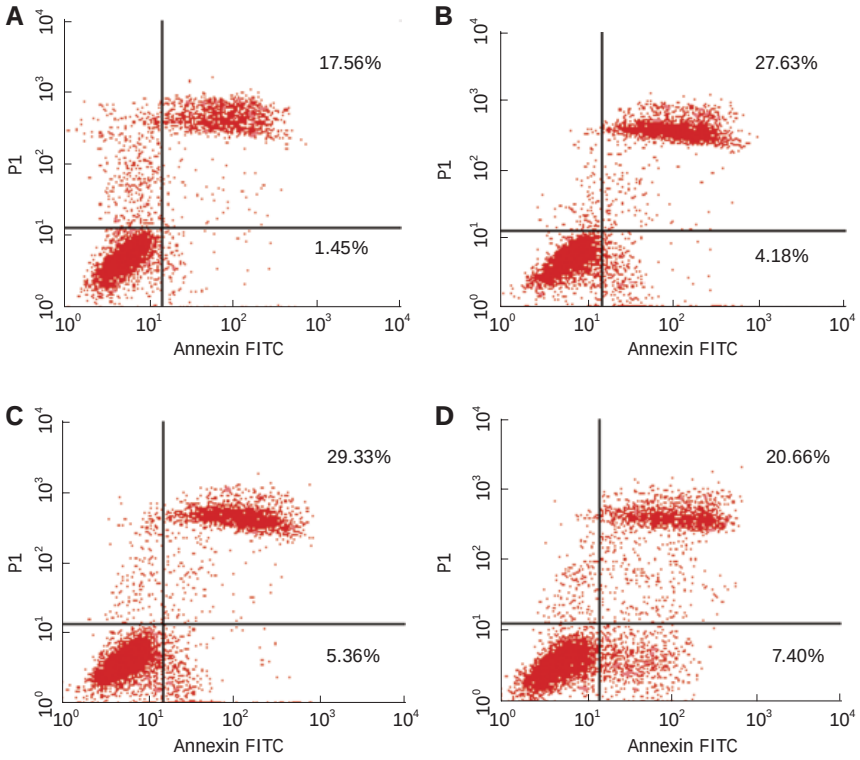

Figure 7 Induction of apoptosis in ECV304 with As_2O_3. Dual-stained with Annexin-V-FITC and PI and analyzed by flow cytometry. There was a dose-dependent increase in early apoptotic cells, as shown in the fourth quadrants plots. **A**: Control; **B**: 0.5 μmol/L As_2O_3; **C**: 2.5 μmol/L As_2O_3; **D**: 5 μmol/L As_2O_3.

the G1 phase.

Tumor angiogenesis appears to be achieved by the overexpression of angiogenic agents within solid tumors that stimulate host vascular EC mitogenesis and possibly chemotaxis. In recent years, it has been widely shown that activity of VEGF is a key feature during tumor growth and angiogenesis[28-30]. In the course of occurrence and development of gastric cancer, angiogenesis plays a crucial role in which VEGF is believed to be the most important factor in neovascularization[31]. Some studies have shown that the serum level of VEGF is not only related to prognosis of gastric cancer, but also to treatment efficacy[32].

Both types of specific receptors for VEGF, Flt-1

and KDR, are commonly distributed in ECs. VEGF acts by binding to the receptors on ECs, and prompts EC proliferation. VEGF and its receptors are the most important pathways in tumor angiogenesis. Inhibition of VEGF/VEGFR pathways may suppress angiogenesis and tumor growth. However, the expression of VEGFR is not EC-specific, and recent emerging evidence has shown that VEGFRs are expressed in several types of non-endothelial cells, especially in tumor cells, which indicates that there is an autocrine pathway of VEGF in tumor cells. A report by Zhang *et al*[8] has shown that eight gastric cancer lines (RF-1, RF-48, NCI-87, NCI-SNU-1, NCI-SNU-5, NCI-SNU-16, AGS-1 and KATO-Ⅲ) express VEGF, and six of these express both Flt-1 and KDR, and exogenous VEGF can stimulate the growth of KDR-positive tumor cells. These results suggest that VEGF acts not only as a paracrine factor on ECs, but also as an autocrine factor on tumor cells. VEGFR may play an important role in paracrine and autocrine pathways of VEGF, and VEGFR inhibitors can inhibit tumor angiogenesis and growth[8,9].

Our recent study[24] has demonstrated that As_2O_3 can inhibit expression of VEGF in gastric cancer. In the present study, we examined further the effect of As_2O_3 on VEGFR expression *in vivo*, and intended to confirm the anticancer activity of arsenic, which may block the paracrine and autocrine VEGF/VEGFR pathways, thereby delaying new tumor blood vessel formation and tumor cell growth. Our results showed that all of the Flt-1 and KDR expressed in the SGC-7901 tumor xenografts, and their expression in tumor cell control were higher than that in arsenic-treated mice. The fluorescence intensity levels of Flt-1 and KDR in tumor cells were significantly reduced in the arsenic-treated groups ($P < 0.001$). The fluorescence intensity levels of Flt-1 and KDR in the 5 mg/kg group were less than those in the 2.5 mg/kg group ($P < 0.001$). These results suggest that As_2O_3 can result in significant down-regulation of Flt-1 and KDR in a dose-dependent manner. The results of further experiments *in vitro* showed that exogenous $VEGF_{165}$ could stimulate the growth of SGC7901 cells, and As_2O_3 may have disturbed the stimulatory effect of $VEGF_{165}$. It indicates that the autocrine pathway of VEGF through VEGFRs is possible in gastric carcinoma, and As_2O_3 inhibits expression of Flt-1 and KDR in endothelial and tumor cells. As_2O_3 may block new blood vessel formation through the paracrine pathway and affect growth of tumor cells through the autocrine pathway of VEGF/VEGFRs, and delay tumor growth.

In conclusion, the results of our present study showed that As_2O_3 delayed growth of human gastric tumor xenografts in nude mice, decreased MVD in tumor tissues, inhibited proliferation of vascular ECs and induced their apoptosis, which resulted in down-regulation of Flt-1 and KDR expression in tumor tissues in a dose-dependent manner. Further results indicate that exogenous $VEGF_{165}$ can stimulate the growth of SGC7901 cells, and As_2O_3 may disturb the stimulatory effect of $VEGF_{165}$. These results suggest that As_2O_3 might delay growth of gastric tumor through inhibiting the paracrine and autocrine pathway of VEGF/VEGFRs.

COMMENTS

Background

Angiogenesis is an important factor in the progression and enlargement of solid neoplasms and is in close relation to invasion and metastases. Inhibition of angiogenesis may lead to control of tumor growth and metastasis, therefore, antiangiogenesis is a promising therapeutic approach for treatment of cancer. VEGF is recognized as one of the most important molecules in the formation of new blood vessels. VEGF binds to two distinct receptors on ECs: Flt-1(VEGFR-1) and KDR (VEGFR-2). Thus, it is suggested that inhibition of VEGF/VEGFR pathways may interrupt VEGF-induced angiogenesis.

Research frontiers

As_2O_3 shows substantial efficacy in treating both newly diagnosed and relapsed patients with APL. Recent studies have shown that a wide variety of malignancies, including both hematological cancer and solid tumors derived from several tissue types, may be susceptible to therapy with As_2O_3. These successes have prompted investigations to elucidate the mechanisms of action underlying these clinical responses. Emerging data suggest that arsenic induces apoptosis, and inhibits tumor growth and angiogenesis. We have recently shown *in vivo* and *in vitro* that As_2O_3 can inhibit VEGF expression and suppress angiogenesis and gastric tumor growth. In the present work, we investigated further the effect of As_2O_3 on expression of VEGF receptors Flt-1 and KDR in human gastric tumor cells and proliferation of vascular ECs.

Innovations and breakthroughs

Our results showed that As_2O_3 delayed growth of human gastric tumor xenografts in nude mice and decreased MVD in tumor tissues, inhibited proliferation of vascular ECs and induced their apoptosis, which resulted in down-regulation of Flt-1 and KDR/Flk-1 expression in tumor tissues in a dose-dependent manner. $VEGF_{165}$ could stimulate the growth of SGC7901 cells, and As_2O_3 may have disturbed the stimulatory effect of $VEGF_{165}$. These results suggest that As_2O_3 might delay growth of gastric tumor through inhibiting the paracrine and autocrine pathway of VEGF/VEGFRs.

Applications

The studies suggest that As_2O_3 might delay growth of gastric tumor through inhibiting the paracrine and autocrine pathway of VEGF/VEGFRs. One of the mechanisms by which As_2O_3 inhibits tumors growth is anti-angiogenesis through inhibiting VEGF/VEGFRs. Our results suggest that As_2O_3 might be used to treat tumors.

Terminology

VEGF is recognized as one of the most important factors in the formation of new blood vessels. As_2O_3 is a common natural substance.

Peer review

This is an excellent paper in terms of scientific rigor, and it is well-written. The experiments were well-designed and well-executed.

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GASTRIC CANCER

Inhibitory effect of schisandrin B on gastric cancer cells *in vitro*

Xiao-Ni Liu, Cheng-Yu Zhang, Xiu-Dong Jin,Yue-Zhen Li, Xue-Zhi Zheng, Li Li

Xiao-Ni Liu, Cheng-Yu Zhang, Xiu-Dong Jin, Yue-Zhen Li, Xue-Zhi Zheng, Li Li, Department of Physiology, Mudanjiang Medical College, Mudanjiang 157011, Heilongjiang Province, China
Supported by The Project of Heilongjiang Natural Science Foundation No. TD 2005-01, and Department of Health, Heilongjiang Province No. 2006-391
Correspondence to: Xiao-Ni Liu, Department of Physiology, Mudanjiang Medical College, Mudanjiang 157011, Heilongjiang Province, China. lxnlxm@126.com
Telephone: +86-453-6582156 Fax: +86-453-6581496
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Abstract

AIM: To investigate the inhibitory effect and possible mechanism of action of schisandrin B in SC-B on gastric cancer cells *in vitro*.

METHODS: SC-B consisted of schisandrin B, aloe-emodin, and *Astragalus* polysaccharides. Exponentially growing human gastric cancer SGC-7901 cells were divided into six treatment groups: (1) control group (RPMI 1640 medium); (2) negative control group (2% DMSO); (3) positive control group (50 mg/L 5-Fluorouracil, 5-FU); (4) low-dose group (LSC, final concentration of schisandrin B, 25 mg/L); (5) moderate-dose group (MSC, final concentration of schisandrin B, 50 mg/L); (6) high-dose group (HSC, final concentration of schisandrin B, 100 mg/L). Follow-up was done at 12-48 h. An MTT (Methylthiazolyldiphenyl-tetrazolium bromide) assay was used to examine the inhibitory effect of SC-B on gastric cancer cells. The mitosis index was assessed using an inverted microscope. Flow cytometry was used to visualize the cell cycle. An RT-PCR (Reverse transcription-Polymerase chain reaction) -based assay was used to detect mRNA expression for cyclin D1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

RESULTS: The MTT assay showed that the number of living cells in the LSC, MSC and HSC groups was significantly smaller than that in the DMSO-treated group ($P < 0.05$) at 12-48 h. The inhibitory rate (IR) of the LSC group was 41.15% ± 3.86%, 59.24% ± 5.34% and 69.93% ± 7.81% at 12, 24 and 48 h, respectively. The IR of the MSC group was 42.82% ± 4.94%, 62.68% ± 7.58% and 71.79% ± 8.12% at 12, 24 and 48 h, respectively. The IR of the HSC group was 37.50% ± 3.21%, 40.34% ± 2.98% and 61.99% ± 4.88% at 12, 24 and 48 h, respectively. These results suggested that a moderate dosage had the most obvious inhibitory efficacy at 48 h. Compared to the DMSO group, the mitosis index of the LSC, MSC, HSC groups was greatly decreased ($P < 0.05$) at all time points. Any dose of SC-B suppressed mitosis within 12-48 h. Compared to the DMSO group, the percentage of cells in the G_0/G_1 phase of the MSC group was greatly increased, and that of the S + G_2M phase was greatly decreased, while the percentage of cell inhibition (PCI) in the MSC group was greatly increased ($P < 0.05$). This suggested that SC-B could exclusively arrest cells in the G_0/G_1 phase. Cyclin D1 mRNA expression was lower in the MSC group than that in the DMSO group ($P < 0.05$).

CONCLUSION: SC-B can inhibit the proliferation and aberrant mitosis of human gastric cancer SCG-7901 cells *in vitro*. This inhibitory effect may be due to the down-regulation of cyclin D1 mRNA expression, which causes cell cycle arrest of gastric cancer cells.

Key words: Aloe-emodin; *Astragalus* polysaccharides; Cell cycle; Cyclin D1; Gastric cancer; Schisandrin B

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INTRODUCTION

Gastric cancer is one of the most prevalent tumors. In China, its incidence and death rate are above those for all other tumors[1]. Therefore, the development of drugs for the treatment of gastric cancer is of great importance. Traditional Chinese medicines and their effective components have certain antitumor effects. This study investigated the inhibitory effect and mechanism of action of SC-B (consisting of schisandrin B, aloe-emodin and Astragalus polysaccharides) on the SGC-7901 cell line *in vitro*.

MATERIALS AND METHODS

Reagents

Schisandrin B and aloe-emodin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Astragalus polysaccharides (UV > 70%) were from East Plant Health Protection. MTT, trypsin (1:250), fetal bovine serum and DMSO were from Sigma (St.

Louis, MO, USA). RPMI 1640 medium and Trizol reagent were from Gibco/BRL (Gaithersburg, MD, USA). Oligo-dT primers, reverse transcriptase (AMV) and Taq polymerase were all from Promega (Madison, WI, USA).

Cell line and cell culture

Human gastric cancer cells (SGC-7901) were purchased from the Department of Cellular and Molecular Biology, Shanghai Institute of Biochemistry and Cell Biology, Academia Sinica. Cells were cultured in MEM containing 10% heated-inactivated fetal calf serum and PNS (100 U/mL penicillin and 10 mL/L streptomycin), at 37℃, in a humidified atmosphere of 95% O_2/5% CO_2. Cells were sub-cultured every 2 or 3 d.

Grouping

Cells were classified into the following groups: control group (treated with RPMI 1640 medium); negative control group (treated with DMSO; final concentration, 2%); positive control group (treated with 5-FU; final concentration, 50 mg/L), low-dose group (LSC, containing 25 mg/L schisandrin B); moderate-dose group (MSC, containing 50 mg/L schisandrin B); and high-dose group (HSC, containing 100 mg/L schisandrin B). Note that SC-B consisted of schisandrin B, aloe-emolin and Astragalus polysaccharides at a ratio of 2:1:1.

MTT experiment

Exponentially growing SGC-7901 cells were digested by 0.25% trypsin for 1-2 min, and washed three times with PBS. RPMI 1640 medium containing 10% newborn bovine serum was added to obtain a cell density of 5×10^8 cells/L. Final cell suspensions (100 μL) were placed in 96-well plates in an incubator containing 5% CO_2, and incubated at 37℃ for 24 h. Then, 100 μL RPMI 1640 medium containing different concentrations of schisandrin B was added to each plate. The effects of schisandrin B were assessed using six replicates for each concentration of schisandrin B. Cells were cultured for 12, 24 and 48 h. The culture medium was changed every 24 h. Four hours before the end of the culture, 20 μL 5 g/L MTT was added to the wells. Optical density (OD) values for each well were measured at 490 nm using an enzyme-linked immunosorbent assay meter. Inhibitory rate (IR) was calculated according to the formula: IR = [1 - (mean of treated group)/(mean of control group)] × 100%.

Mitosis index

Cellular mitosis was observed using an inverted microscope, and the proportion of mitotic cells in 1000 cells was calculated after SC-B was added to the plates. The drug concentrations and incubation times were the same as those used in the MTT experiment.

Cell cycle analysis

Exponentially growing SGC-7901 cells were used for this experiment, and the cell density was adjusted to 3×10^8 cells/L by adding RPMI 1640 medium containing 10% newborn bovine serum, to obtain a 2 mL final volume in each six-well plate. Six-well plates were placed at 37℃ in an incubator containing 5% CO_2 for 24 h. The culture medium was changed 24 h later. Once the 2 mL final cell suspensions (containing SC-B with a final concentration of schisandrin B of 50 mg/L) were added, the cells were left to incubate for 48 h. Finally, samples were processed to obtain simple cell suspensions (target cell number was > 5×10^9 cells/L), which were fixed with 70% cold ethanol for 24 h, after centrifugation for 5 min at 200 × *g*. The cells were further centrifuged for 15 min at 200 × *g*, and then resuspended in 0.5 mL PBS. Then, they were digested by Rnase-A (final concentration, 50 mg/L) for 30 min at 37°C, and stained with propidium iodide (final concentration, 65 mg/L) for 30 min. Finally, cell suspensions were analyzed for the cell cycle using a nylon monofilament mesh screen with 44-μm openings and flow cytometry. The percentage of cell inhibition (PCI) was calculated according to the formula: PCI = [(Percentage of S + G_2M in DMSO group - Percentage of S + G_2M in treatment group) / Percentage of S+G_2M in DMSO group] × 100%.

Detection of Cyclin D1 mRNA

The methods for cell culture, including drug concentrations and incubation times were the same as those for the cell cycle analysis.

Total mRNA extraction

Cells in each six-well plate were homogenized in 1 mL Trizol and 0.2 mL chloroform, and the mixture was placed on ice for 5 min. After sedimentation by centrifugation at 12000 × *g* at 4℃, RNA was precipitated by combining the aqueous phase with an equal volume of isopropanol. The precipitate was recovered by centrifugation (12000 × *g* at 4℃), washed once with 75% ethanol, and solubilized in diethylpyrocarbonate (DEPC)-treated water. RNA concentrations were measured with a UV spectrometer. RNA/DNA (A_{260}/A_{280}) ratio had to be > 1.8.

Reverse transcription (RT)

Each RT reaction consisted of 1 μg extracted RNA, 1 μL 10 × RT buffer, 4 μL dNTPs (10 mmol/L), 0.5 μL oligo-dT primers, 1 μL AMV, and 0.25 μL RNase inhibitor, and DEPC-treated water was added to obtain a final reaction volume of 10 μL. RT was initially performed at 95℃ for 3 min, and placed on ice. Other reactants were added to the reaction system, and the mixture was further incubated at 42℃ for 70 min, and held at 95℃ for 3 min. Samples were frozen for later use.

Polymerase chain reaction (PCR)

Each PCR reaction included 10 μL 5 × PCR Buffer, 1.6 μL dNTPs, 0.5 μL of a 25 μmol/L solution of each primer, 0.25 μL Taq DNA polymerase, 10 μL RT products, and DEPC-treated water. The total reaction volume was 40 μL. PCR reaction conditions are shown in Table 1. The amplified products were visualized by electrophoresis on a 1.5% agarose gel, stained with 1 mg/L ethidium bromide, and illuminated and analyzed with a UVP gel imaging system (BIO-PRO, Carlsbad, CA, USA).

Table 1 Primers and PCR conditions

mRNA	Primer (5′-3′)	Primer size (bp)	PCR conditions
CyclinD1	F: TGGATGCTGGAGGTCTGCGAGGAA R: GGCTTCGATCTGCTCCTGGCAGGC	573	95℃/3 min;
GAPDH	F: GAAGGTGAAGGTCGGAGT R: GA AGATGGTGATGGGATTTA	320	(94℃/30 s; 55℃/30 s; 72℃/60 s) 35 cycles

F: Forward; R: Reverse.

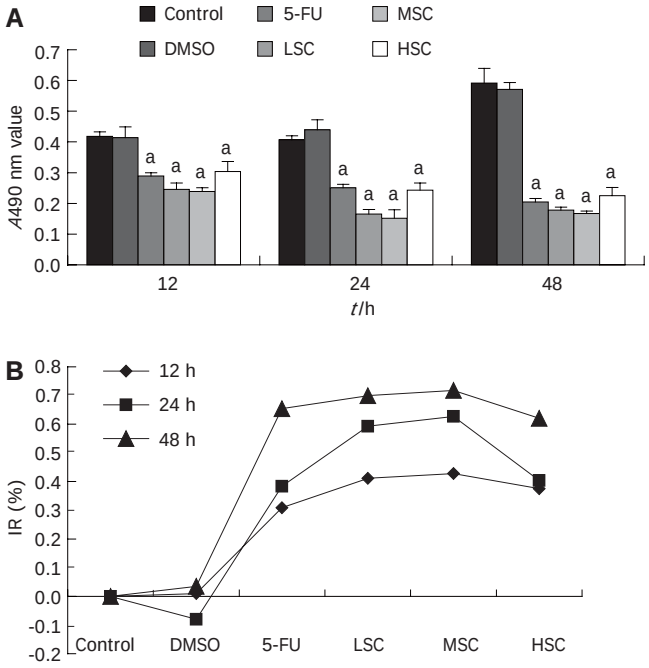

Figure 1 Effect of SC-B on gastric cancer SGC-7901 cell proliferation. Gastric cancer SGC-7901 cells were treated with SC-B (final concentration of schisandrin B was 25 mg/L, 50 mg/L and 100 mg/L for the LSC, MSC, and HSC groups, respectively) at the indicated times. **A**: Optical density values at 490 nm for each group were measured; **B**: Inhibitory rates (IR) of the 3 dosages are shown at different time points. $^aP < 0.05$ *vs* DMSO group.

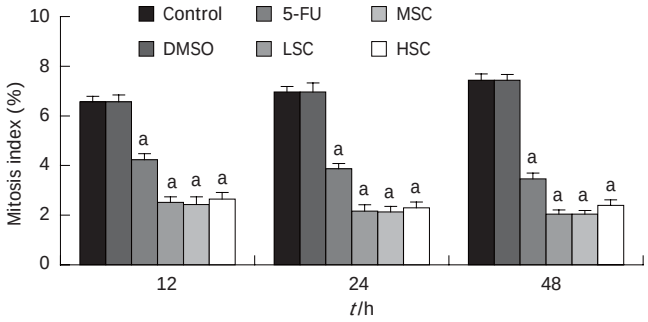

Figure 2 Influence of SC-B on the mitosis index of SGC-7901 cells. Gastric cancer SGC-7901 cells were treated with SC-B (final concentration of schisandrin B was 25 mg/L, 50 mg/L, and 100 mg/L for the LSC, MSC, and MSC groups, respectively) at the indicated times; $^aP < 0.05$ *vs* DMSO group.

Statistical analysis

Data were processed using SPSS10.0 software. One-way ANOVA analysis was used to examine the differences between the groups.

RESULTS

Effect of SC-B on gastric cancer SGC-7901 cells

Our results showed that the living cell numbers in the LSC, MSC, HSC groups were significantly smaller than that in the DMSO group ($P < 0.05$), as measured by the MTT assay, at 12, 24 and 48 h. The IR of the LSC group was 41.15% ± 3.86%, 59.24% ± 5.34%, and 69.93% ± 7.81% at 12, 24 and 48 h, respectively. The IR of the MSC group was 42.82% ± 4.94%, 62.68% ± 7.58% and 71.79% ± 8.12% at 12, 24 and 48 h, respectively. The IR of the HSC group was 37.50% ± 3.21%, 40.34% ± 2.98% and 61.99% ± 4.88% at 12, 24 and 48 h, respectively. These results suggested that a moderate dosage of SC-B had the most obvious inhibitory effect at 48 h (Figure 1).

Influence of SC-B on the mitosis of gastric cancer SGC-7901 cells

Compared to the DMSO group, the mitosis index of the LSC, MSC, HSC groups was greatly decreased ($P < 0.05$) at different time points. This suggested that SC-B could suppress mitosis of SGC-7901 cells treated with the three doses within 12 to 48 h (Figure 2).

Influence of SC-B on the cell cycle of gastric cancer SGC-7901 cells

Compared to the DMSO group, in the MSC group, the number of cells in the G_0/G_1 phase was greatly increased (62.59% ± 2.42% *vs* 56.76% ± 2.56%), but the number in the S + G_2M phase was greatly decreased (37.42% ± 2.42% *vs* 43.25% ± 2.56%). The PCI of the MSC group was greatly increased compared to the DMSO group (20.42% ± 3.11% *vs* 7.29% ± 2.83%, $P < 0.05$). This suggested that SC-B could exclusively arrest cells in the G_0/G_1 phase (Figures 3 and 4).

Influence of SC-B on cyclin D1 mRNA expression of gastric cancer SGC-7901 cells

GAPDH mRNA was used as the standard for PCR (320 bp). The ratio of cyclin D1 mRNA to GAPDH mRNA was used to quantify the expression level of cyclin D1 mRNA (573 bp). The results showed that cyclin D1 mRNA expression in the MSC group was lower than that in the DMSO group ($P < 0.05$) (Figure 5).

DISCUSSION

Schisandrin B, extracted from the fruit of *Schisandra chinensis* Baill, has been reported to have antitumor

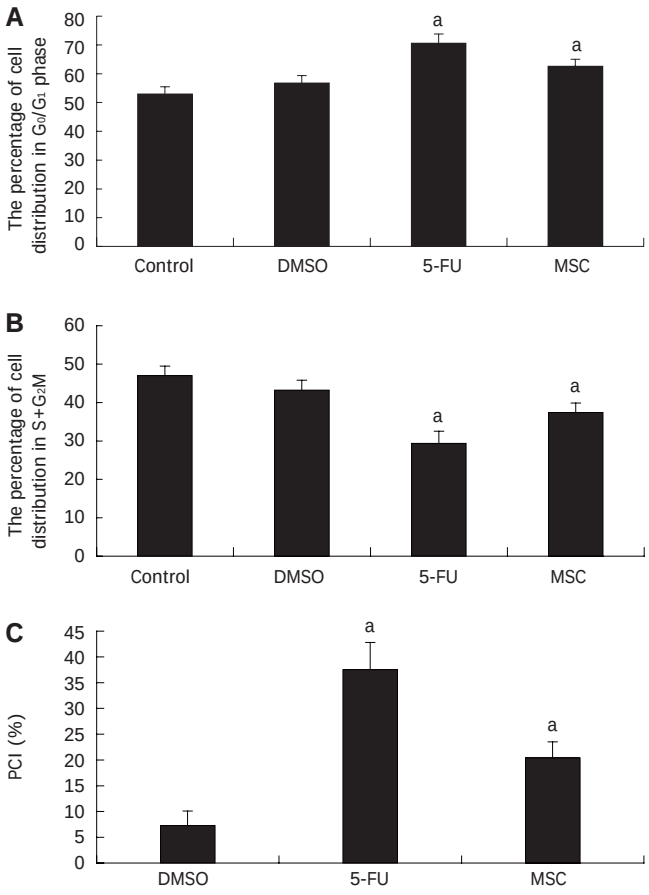

Figure 3 Effect of SC-B on the cell cycle of gastric cancer SGC-7901 cells. Gastric cancer SGC-7901 cells were treated with SC-B (final concentration of schisandrin B, 50 mg/L) for 48 h. **A**: Percentage of cell distribution in the G_0/G_1 phase; **B**: Percentage of cell distribution in the S+G_2M phase; **C**: Percentage of cell inhibition (PCI). $^aP < 0.05$ *vs* DMSO group.

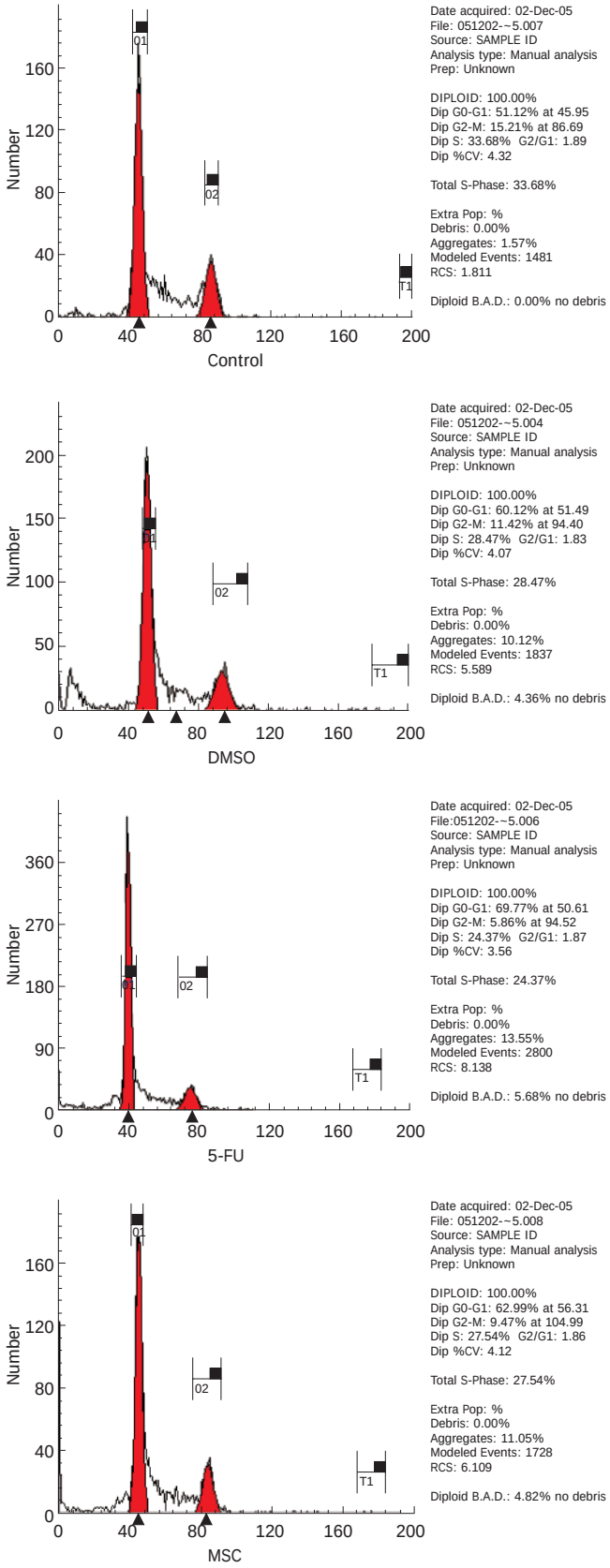

Figure 4 Flow cytometry results showing the influence of SC-B on the cell cycle of gastric cancer SGC-7901 cells.

effects. For example, schisandrin B can inhibit the proliferation of human hepatoma SMMC-7721 cells and induce apoptosis, involving the caspase-3-dependent and caspase-9-independent pathways, accompanied by down-regulation of Hsp70 protein expression at an early stage[2]. Schisandrin B enhances doxorubicin-induced apoptosis of SMMC7721 cells and MCF-7, a human breast cancer cell line, but not normal cells. This enhancement is associated with the activation of caspase 9[3]. Schisandrin B inhibits DNA synthesis in ascitic hepatoma cells[4]. Aloe-emodin is purified from *Aloe vera* leaves. Aloe-emodin modulates protein kinase C isozymes, inhibits proliferation, and induces apoptosis in U-373MG glioma cells[5]. The aloe-emodin-induced increase in the amount of proforms and fragments of nucleophosmin in the cytoplasm may be one of the important events for aloe-emodin-induced H460 cell apoptosis[6]. All this information supports the hypothesis that aloe-emodin represents a novel antitumor chemotherapeutic drug. *Astragalus mongholicus* polysaccharides have been isolated from one of the Chinese herbs, and are known to have various immunomodulatory activities[7]. We have previously shown that schisandrin B and aloe-emodin clearly inhibit the multiplication of human SGC-7901 cells within 12 to 48 h, and we have found that the most effective concentration ratio of schisandrin, aloe-emodin and *Astragalus* polysaccharides in SC-B is 2:1:1. The inhibitory effect of SC-B is significantly higher than that of schisandrin, aloe-emodin and *Astragalus* polysaccharides on their own[8].

This study investigated the inhibitory effect and

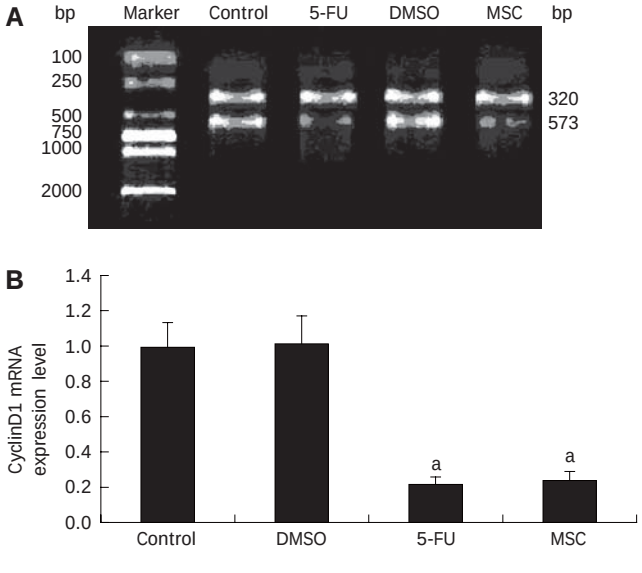

Figure 5 Influence of SC-B on cyclinD1 mRNA expression in gastric cancer SGC-7901 cells. Gastric cancer SGC-7901 cells were treated with SC-B (final concentration of schisandrin B, 50 mg/L) for 48 h. **A**: Electrophoretic bands of cyclinD1 mRNA and GAPDH; **B**: Half-quantitative results of the expression level of cyclinD1 mRNA, $^aP < 0.05$ *vs* DMSO group.

mechanism of SC-B on the SGC-7901 gastric cancer cell line *in vitro*. We showed that SC-B (25-100 mg/L) clearly inhibited the multiplication and mitosis of human gastric cancer SGC-7901 cells within 12-48 h, but a dose of 50 mg/L SC-B had the strongest inhibitory effect at 48 h. Therefore, when we further investigated the possible mechanisms of SC-B, we decided to use the moderate concentration (50 mg/L) and the 48-h time point.

The cell cycle consists of the G_1 phase (growth and preparation of the chromosomes for replication), S phase (synthesis of DNA), G_2 phase (preparation for mitosis), and M phase (mitosis). A eukaryotic cell cannot divide into two, and the resulting two cells cannot divide into four, unless two processes are alternated. These include the doubling of the DNA genome in the S phase (synthesis phase), and the halving of this genome during mitosis (M phase). The period between M and S is called the G_1 phase, and the period between S and M is the G_2 phase. Sometimes, a cell leaves the cell cycle temporarily or permanently. It exits the cycle at G_1, and enters a stage designated G_0. G_0 represents the absence of signals for mitosis, and an active repression of the genes needed for mitosis. Cancer cells cannot enter G_0, and are destined to repeat the cell cycle indefinitely[9]. If some mechanism can prevent S phase cancer cells from entering the M phase, then the infinite growth of cancer cells could be controlled. In our study, exponentially growing cells were used to analyze the changes in the cell cycle. We showed that SC-B (50 mg/L) exclusively arrested cells in the G_0/G_1 phase compared to the DMSO group. The number of cells in the G_0/G_1 phase in the MSC group was greatly increased ($P < 0.05$ *vs* DMSO group), which suggests that SC-B (50 mg/L) can prevent G_1 phase cancer cells from entering the M phase.

Quality controls of the cell cycle are cell-cycle checkpoints. Some key checkpoints are: G_1 checkpoint (to assess DNA damage before the cells enter S phase); S checkpoint (to monitor the presence of the Okazaki fragments on the lagging strand during DNA replication); G_2 checkpoint (during S phase, and after DNA replication); and M checkpoint (to detect any failure of spindle fibers to attach to the kinetochores, and arrest the cell in metaphase)[10-14]. Checkpoints can become activated due to DNA damage, exogenous stress signals, defects during DNA replication, or failure of the chromosomes to attach to the mitotic spindle[15]. Whether the cell cycle starts to allow the cells to proliferate depends on the G_1 checkpoint, which determines if the cells enter the S phase. The cell cycle is controlled by proteins in the cytoplasm. One of the main players in animal cells is cyclin D1. This is one of the important regulative factors that can induce cells from G_1 to enter the S phase. Increased amounts of cyclin D1 bind to cyclin-dependent kinases 4/6, which is the signal to prepare the chromosomes for replication[16,17]. RT-PCR was used to measure cyclin D1 mRNA expression in this study. The results showed that cyclin D1 mRNA expression was significantly reduced in the MSC group (50 mg/L) compared to the DMSO group ($P < 0.05$). This suggested that SC-B suppressed the multiplication and aberrant mitosis of human gastric cancer SGC-7901 cells by reducing cyclin D1 mRNA expression, which caused the cell cycle to arrest by inhibiting the G_1 checkpoint (i.e. by preventing gastric cancer cells from G_1 entering the S phase).

COMMENTS

Background

Gastric cancer is one of the most prevalent malignant tumors, and is classified as one of the first and most severe form of cancer harming people's health. Therefore, the exploitation of drugs to treat gastric cancer is of great significance. Traditional Chinese medicines and their effective components have certain antitumor effects.

Research frontiers

Schisandrin B, a dibenzocyclooctadiene derivative was isolated from the fruit of Schisandra chinensis. Many studies showed that schisandrin B has anticancer effects. Now, studies on the application of Schisandrin B in the preparation of anticancer medications, particularly for the treatment of multidrug resistant (MDR) cancer are hot research topics. Schisandrin B effectively reverses MDR cancer in combination with other anticancer chemotherapeutic agents.

Innovations and breakthroughs

Many other researches have revealed that Schisandrin B has anticancer effects, but Schisandrin B is cytotoxic to human cancer cells, which restricts its application in cancer chemotherapy. In this study, we successfully prepared SC-B for human use by mixing schisandrin B with aloe-emodin and Astragalus polysaccharides. The anticancer effect of SC-B was greatly increased compared with that of Schisandrin B on its own. In this study, we observed the influence of SC-B on living cell numbers, mitosis index, cell cycle, and $CyclinD_1$ expression in SGC-7901 cells. The results suggested that SC-B could suppress the multiplication and aberrant mitosis of human gastric cancer SGC-7901 cells by reducing $CyclinD_1$ mRNA expression, causing cell-cycle arrest by inhibiting the G_1 checkpoint.

Applications

SC-B could be applied in the treatment of gastric cancer, and might provide a potential therapeutic anticancer drug.

Terminology

Schisandrin B: Schisandrin B is a dibenzocyclooctadiene derivative isolated from the fruit of *Schisandra chinensis and Schisandra chinensis,* a woody vine which

bears numerous clusters of tiny, bright red berries; Cell cycle: The cell cycle or cell-division cycle refers to the series of events that take place in a eukaryotic cell from its formation and the moment it replicates itself; Cyclins: Cyclins are a family of proteins involved in the progression of cells through the cell cycle. The main players in animal cells are: G1 cyclin (cyclin D), S-phase cyclin (cyclins E and A), and mitotic cyclins (cyclins B and A).

Peer review

This is a good descriptive report on the potential chemotherapeutic effects of schisandrin B in gastric cancer, *in vitro*.

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COLORECTAL CANCER

New anti-proliferative agent, MK615, from Japanese apricot "*Prunus mume*" induces striking autophagy in colon cancer cells *in vitro*

Shozo Mori, Tokihiko Sawada, Toshie Okada, Tatsushi Ohsawa, Masakazu Adachi, Kubota Keiichi

Shozo Mori, Tokihiko Sawada, Toshie Okada, Kubota Keiichi, Second Department of Surgery, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan
Tatsushi Ohsawa, Masakazu Adachi, Japan Apricot Co., Ltd., Midori-cho 1-11-7, Takasaki, Gunma 370-0073, Japan
Correspondence to: Tokihiko Sawada, MD, PhD, Second Department of Surgery, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan. tsawada@dokkyomed.ac.jp
Telephone: +81-282-861111 Fax: +81-282-866317
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Abstract

AIM: To investigate the anti-neoplastic effects of MK615, an extract from the Japanese apricot (*Prunus mume*), against colon cancer cells.

METHODS: Three colon cancer cell lines, SW480, COLO, and WiDr, were cultured with MK615. Growth inhibition was evaluated by cell proliferation assay and killing activity was determined by lactate dehydrogenase assay. Induction of apoptosis was evaluated by annexin V flow cytometry. Morphological changes were studied by light and electron microscopy, and immunofluorescence staining with Atg8.

RESULTS: MK615 inhibited growth and lysed SW480, COLO and WiDr cells in a dose-dependent manner. Annexin V flow cytometry showed that MK615 induced apoptosis after 6 h incubation, at which point the occurrence of apoptotic cells was 68.0%, 65.7% and 64.7% for SW480, COLO, and WiDr cells, respectively. Light and electron microscopy, and immunofluorescence staining with Atg8 revealed that MK615 induced massive cytoplasmic vacuoles (autophagosomes) in all three cell lines.

CONCLUSION: MK615 has an anti-neoplastic effect against colon cancer cells. The effect may be exerted by induction of apoptosis and autophagy.

Key words: Colon cancer; Japanese apricot; *Prunus mume*; Autophagy; Apoptosis; MK615

Mori S, Sawada T, Okada T, Ohsawa T, Adachi M, Keiichi K.

INTRODUCTION

Japanese apricot, *Prunus mume* Sieb. et Zucc. (known as *ume* in Japanese), has for centuries been a traditional Japanese medicine, and is a familiar and commonly consumed food. *Prunus mume* is a species of Asian plum of the family Rosaceae. It is often called a plum, but it is more closely related to the apricot (ume in Japanese). MK615, an extract of compounds from Japanese apricot, has been shown to possess an anti-proliferative effect against some cancer cell lines[1,2]. Although MK615 induces apoptosis of breast cancer cells and some other human cancer cells, morphological studies have revealed the appearance of abundant cytoplasmic vacuoles early after MK615 exposure, which are not relevant to apoptosis[2]. The precise role of these vacuoles has not been elucidated.

Even though effective chemotherapeutic agents and regimens have been developed, colorectal cancer is still associated with high rates of morbidity and mortality worldwide[3,4]. Furthermore, the side effects of chemotherapeutic agents often hamper the quality of life of patients with colorectal cancer. There is a need, therefore, to develop new, effective and less toxic chemotherapeutic agents against colorectal cancer.

In the present study, we investigated the antineoplastic effects of MK615 against colon cancer cell lines *in vitro*. We found that MK615 strongly induced autophagy in colon cancer cells and exerted antineoplastic effects.

MATERIALS AND METHODS

MK615, derived from the fruit of the Japanese apricot[1], was provided by the Japan Apricot Co. (Gunma, Japan). The preparation involved the extraction of apricot juice using a press. This was then heated and concentrated. The concentrate was dissolved in diethylether, which was then completely removed from the extract by rotary evaporation. The dried hydrophobic extract, MK615, was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industry, Osaka, Japan) at several concentrations.

The MK615/DMSO solution was applied to cell cultures at a concentration of 10 μL/mL of culture medium. DMSO alone was added as a negative control.

Cells

Three human colon cancer cell lines, SW480, COLO, and WiDr, were purchased from the American Type Culture Collection (Manassas, VA, USA). These three were selected because of the differences in sex, race, and the productivity of proteins, such as carcinoembryonic antigen (CEA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) in a 5% CO_2 incubator.

Cell proliferation assay

The cells were plated at 1×10^4/well in 96-well plates in DMEM containing 10% (v/v) FCS. The cells were then cultured with or without MK615 at concentrations of 150, 300 or 600 μg/mL. For negative control wells, cells were cultured with 1% DMSO alone. After 48 h, 15 μL MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) (5 g/L) was added to each well, and the cells were incubated for 4 h. Then, 100 μL solubilization solution/stop mix was added following the manufacturer's instructions (Promega, Madison, WI, USA), and the plates were left to stand for 60 min. Absorbances at 570 nm and 630 nm were measured with an ELISA reader. Actual counts were calculated by subtracting the absorbance at 570 nm from that at 630 nm. Each assay was performed in triplicate and the average absorbance was calculated. The data presented here were calculated using the ratio of absorbance at each drug concentration to the absorbance in the absence of drugs.

Lactate dehydrogenase (LDH) assay

LDH assay was performed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Briefly, cells were plated at 5×10^3/well in 96-well plates in DMEM containing 10% (v/v) FCS, then cultured with or without MK615 at 150, 300 or 600 μg/mL. For the negative control wells, cells were cultured with 1% DMSO alone. The medium was removed and 100 μL CytoTox-ONE Reagent was added. Cells were then incubated at 22℃ for 10 min and 50 μL stop solution added. The plates were shaken for 10 s and the absorbances at 560 nm and 590 nm measured with an ELISA reader. To obtain the maximum LDH release, 10 μL lysis solution (10 ×) was added to the positive control wells 45 min prior to harvest. After 48 h, a 50 μL supernatant was transferred to a fresh 96-well plate, and incubated for 30 min with 50 μL substrate mix at room temperature. Then, 50 μL stop solution was added, and the absorbance at 490 nm measured with an ELISA reader. Each assay was performed in triplicate and the average absorbance calculated. The data presented here were calculated using the formula: percentage cytotoxicity = 100 × (experimental - culture medium background) /(maximum LDH release - culture medium and lysis solution background).

Flow cytometry

Cells were cultured with MK615 at a concentration of 300 μg/mL for 6 h, then harvested by trypsinization and washed twice with PBS. Annexin V binding assay was performed using the Annexin V-FITC Apoptosis Detection kit (Becton-Dickinson, NJ) according to the supplier's instructions. At least 1×10^6 cells were incubated with FITC-conjugated annexin V at room temperature for 15 min. Cells were then analyzed on a FACScalibur flowcytometer (Becton-Dickinson).

Light microscopy

Cells were cultured with MK615 at a concentration of 300 μg/mL for 6 h, then collected by trypsinization. Collected cells were mounted on glass slides by the spin-down procedure (50 rpm, 3 min) and stained with Diff Quick staining solution (International Reagents, Kobe, Japan). Morphological changes were assessed by light microscopy of Diff Quick specimens.

Electron microscopy

Cells were cultured with MK615 at concentrations of 150, 300 or 600 μg/mL for 6 h, then fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), followed by 1% osmium tetroxide. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation using a JEM 100 CX electron microscope (JEOL, Peabody, NY, USA).

Immunofluorescence staining

Cells were cultured with MK615 at a concentration of 300 μg/mL for 6 h, then fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. They were then washed three times in PBS containing 0.01% Triton X-100 and 10% FBS, followed by incubation with anti-autophagy cleaved-APG8b (MAPILC3B) antibody (ABGENT, CA, USA), or 4', 6-diamino-2-phenylindole (DAPI) nucleic acid stain (Lonza, Basel, Switzerland).

Statistical analysis

Statistical analysis for the cell proliferation assay and LDH assay was performed by Student's *t* test, comparing the counts at 0 μg/mL MK615 with those at each concentration of MK615. The percentage inhibition was calculated using the ratio of absorbance at each concentration of MK615 relative to the absorbance with no drug added.

RESULTS

The anti-proliferative effects of MK615 against colon cancer cells were evaluated by cell proliferation assay (Figure 1). The percentage inhibition rates of SW480 at 150, 300 and 600 μg/mL MK615 were 31.9%, 58.5% and 54.2%, respectively, and those of COLO and WiDr were 38.9%, 70.4% and 72.4%, and 12.1%, 54.5% and 58.3%, respectively. The inhibition induced by MK615 at all concentrations was significantly higher than that in the absence of MK615, and was dose-dependent in the COLO and WiDr cell lines.

We examined the ability of MK615 to kill colon cancer cells using a LDH-releasing assay (Figure 2). The percentage specific lysis of SW480 at 0, 150, 300 and 600 μg/mL

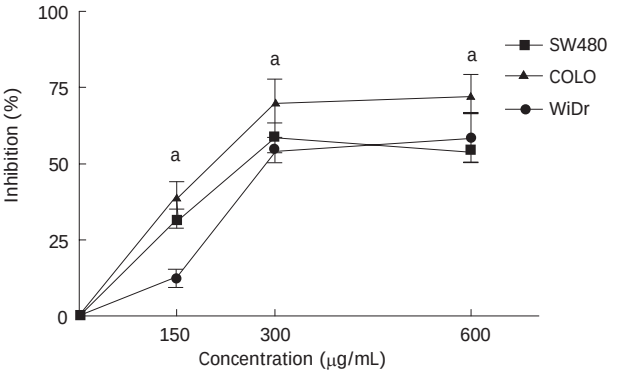

Figure 1 Dose-dependent inhibition of colon cancer cell growth by MK615. Growth inhibition was evaluated by MTT assay. The percentage inhibition (Y axis) was calculated using the ratio of absorbance at each drug concentration relative to absorbance in absence of the drugs. $^{a}P < 0.05$.

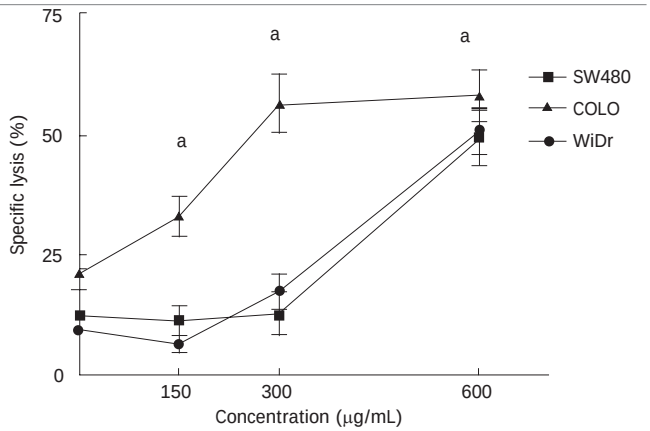

Figure 2 Dose-dependent lysis of colon cancer cells by MK615. Cells were challenged with 150, 300 or 600 μg/mL MK615. All three colon cancer cell lines were lysed effectively in a dose-dependent manner. $^{a}P < 0.05$.

MK615 was 12.5%, 11.3%, 12.8% and 49.6%, respectively. The respective values for COLO and WiDr were 21.4%, 33.1%, 56.6 and 58.3%, and 9.4%, 6.4%, 17.4% and 50.7%. The percentage specific lysis at all concentrations was significantly higher than that for the controls in all three cell lines.

The antiproliferative effect of MK615 is partly attributed to the induction of apoptosis[2]. As shown in Figure 3, MK615 treatment induced apoptosis in all three colon cancer cell lines. After incubation with 300 μg/mL MK615 for 6 h, the frequencies of apoptotic cells in SW480, COLO and WiDr cells were 68.0%, 65.7% and 64.7%, respectively.

It has also been reported that MK615 treatment induces the formation of cytoplasmic vacuoles in breast cancer cells[2]. Similarly, in the present study with colon cancer cells, abundant cytoplasmic vacuoles were observed in all three cell lines after 6 h incubation with MK615 at 300 μg/mL (Figure 4). Electron microscopy revealed that the cytoplasmic vacuoles were typical autophagosomes. Although some cells showed typical features of apoptosis (Figure 5A), there were abundant autophagosomes showing a membrane structure within which cytoplasmic structures were entrapped (Figure 5B-F). In some cells, degeneration of mitochondria was observed (Figure 5).

Immunofluorescence staining with Atg8 (LC3) showed positive labeling in all three cell lines after treatment with MK615 (Figure 6). Nuclei were stained blue with DAPI (Figure 6 A, C and E), and Atg8 (LC3) produced peripheral red staining (Figure 6 B, D and F).

DISCUSSION

In the present study, we showed that MK615 inhibited the growth of three colon cancer cell lines, SW480, COLO, and WiDr, and induced massive autophagy. Apoptosis is a well known form of programmed cell death (PCD), and is widely accepted as the main mechanism of cancer cell death[5-8]. Currently, apoptosis is classified as a type I PCD, whereas autophagy is classified as type II PCD[9-13]. Autophagy differs in several ways from type I PCD. Autophagy is a form of caspase-independent cell death, displays no DNA-laddering, and is typically characterized by the formation of cytoplasmic vacuoles. In recent years, the importance of autophagy has been stressed in various biological fields, including cancer[14-16]. Autophagy is an evolutionarily conserved pathway that delivers and recycles cytoplasmic components, such as mitochondria and Golgi apparatus. Thus, starvation is a typical trigger that can induce autophagy. Some studies have indicated that cancer cells show less autophagy than normal cells[17,18], which suggests that induction of autophagy is an attractive mode of anti-cancer therapy.

Autophagy can be induced by various agents, such as tamoxifen[19], γ-irradiation[20,21], and rapamycin[22,23], but few studies have investigated the induction of autophagy by natural substances. A previous study has found that soybean B-group triterpenoid saponins induce macroautophagy in human colon cancer cells[24].

Atg8 is a 117-amino-acid protein essential for the early phase of autophagy[25]. LC3 is a mammalian homologue of Atg8 and is the only established marker of autophagy in mammalian cells[26]. Conditions such as nutrient starvation initiate the formation of a double-membrane structure, which elongates to form a cytoplasmic vesicle, the autophagosome. Finally, autophagosomes fused with lysosomes generate single-membrane autophagolysosomes, resulting in the degradation of their content. Atg8 (LC3) binds to the membrane of the autophagosome and plays a crucial role in the process of autophagy[27,28]. In the present study, MK615 induced the expression of Atg8 (LC3) in all the colon cancer cell lines within 6 h of incubation. Expression of Atg8 (LC3) was exclusively localized in the cytoplasm. These findings indicated that MK615 induced massive autophagy in colon cancer cells *in vitro*.

Precise elucidation of the mechanism responsible for the antineoplastic effect of MK615 will require further study, as it remains unclear whether autophagy suppresses tumorigenesis or provides cancer cells with a rescue mechanism under unfavorable conditions. Kondo *et al* have reported that manipulation of autophagy may have different effects in different types of cancer cells. If cancer cells show defective autophagy, induction of autophagy

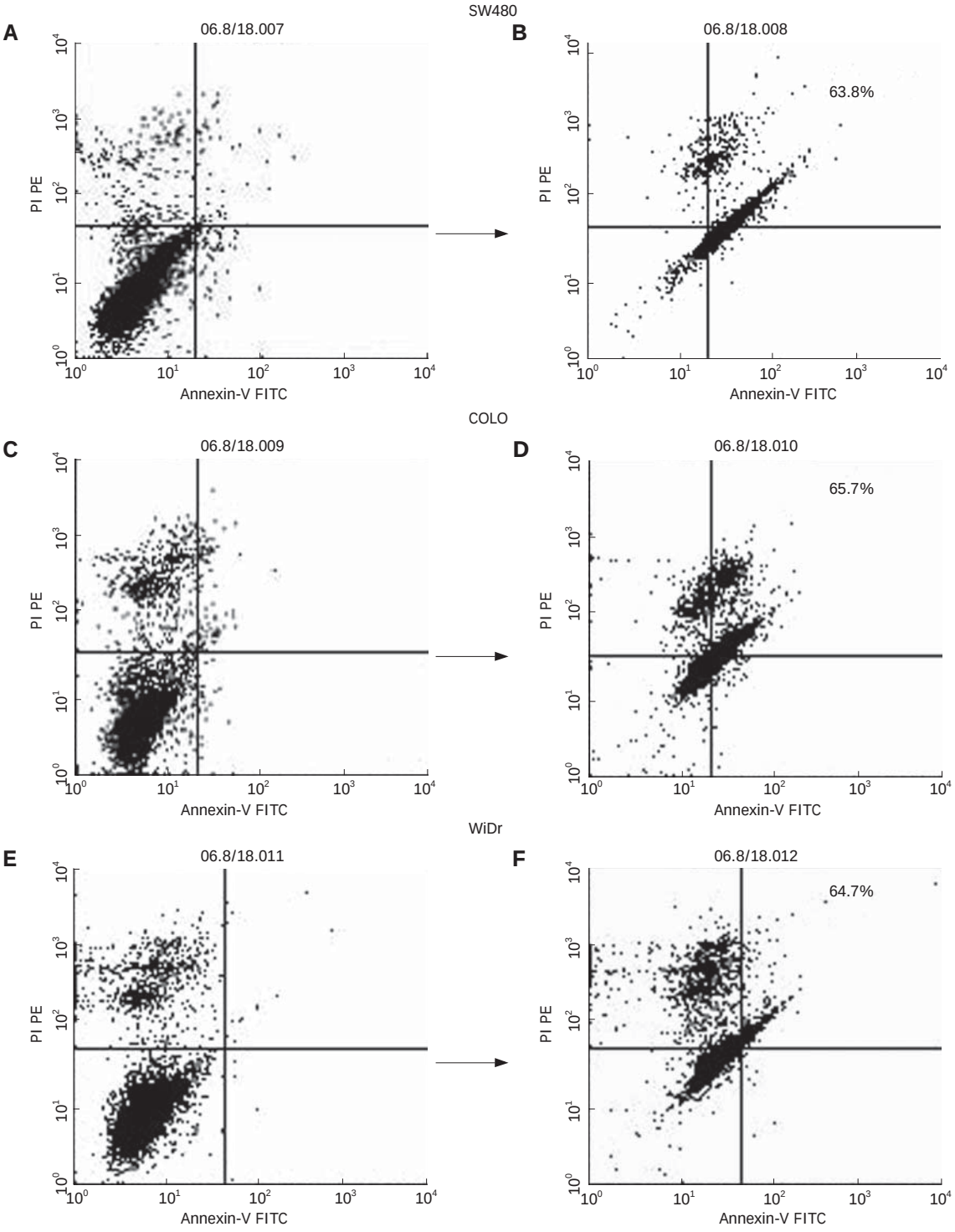

Figure 3 MK615-induced apoptosis in colon cancer cell lines. SW480, COLO and WiDr cells were cultured without (**A**, **C** and **E**) and with (**B**, **D** and **F**) MK615 at 300 mL, and harvested after 6 h incubation.

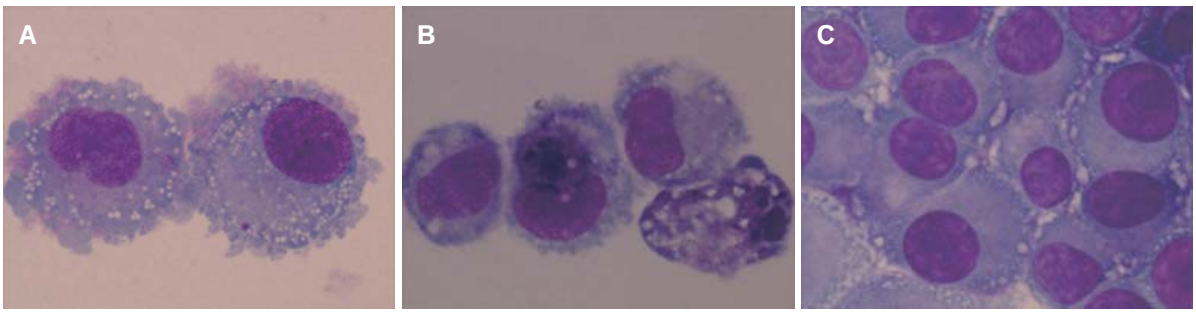

Figure 4 Massive induction of cytoplasmic vacuoles by MK615. MK615 (300 μg/mL) induced cytoplasmic vacuoles in SW480 (**A**), COLO (**B**) and WiDr (**C**) after 6 h incubation.

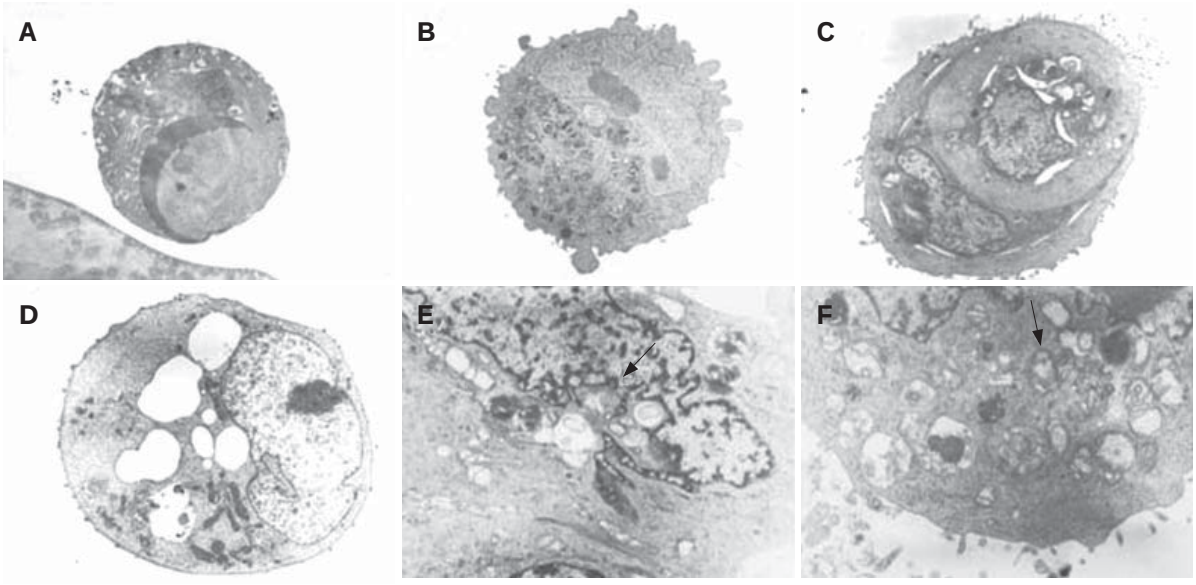

Figure 5 Electron micrographs of autophagy induced by MK615. **A**: MK615 induced typical features of apoptosis in SW480 cells; **B-F**: Autophagy induced by MK615. Cytoplasmic vacuoles (autophagosomes) induced by MK615 in SW480 **B** and **C**, COLO **D** and **E** and WiDr (**F**) cells. Degenerated mitochondria are evident (arrows in **E** and **F**).

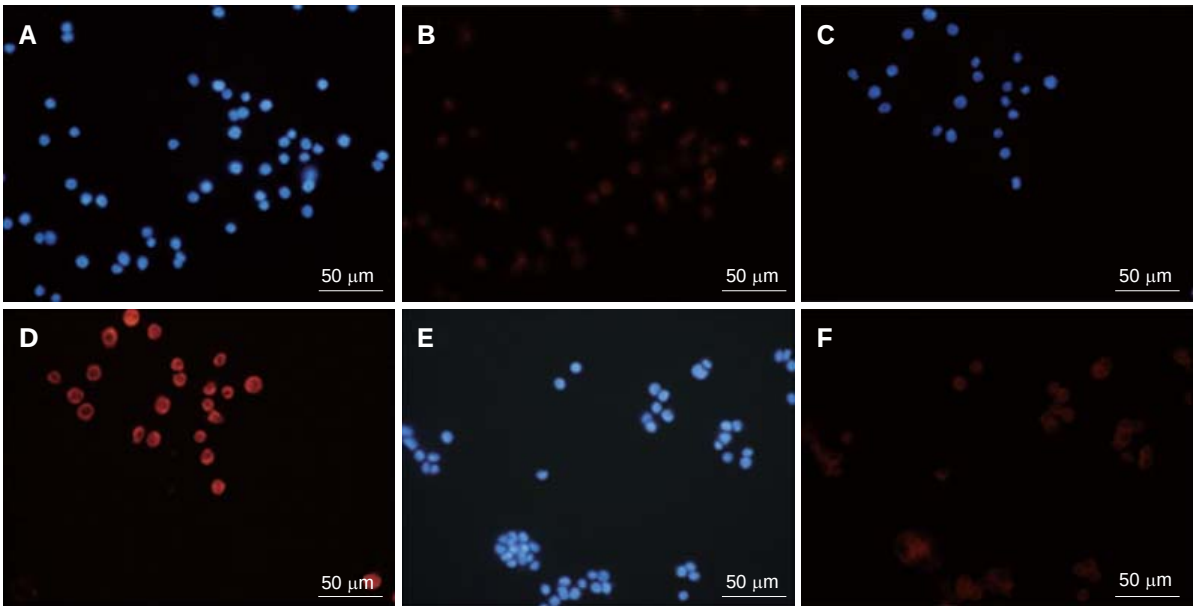

Figure 6 Immunofluorescence staining with Atg8 (LC3). SW480 (**A**), COLO (**B**) and WiDr (**C**) cells were cultured with MK615 at 300 μg/mL for 6 h. Nuclei were stained with DAPI (**A**, **C** and **E**), and Atg8 (LC3) was localized in the cytoplasm (**B**, **D** and **F**; note the central blank area in the cells).

may lead to the inhibition of cancer growth, whereas if cancer cells show protective autophagy in response to chemotherapy, this may decrease their sensitivity to anti-cancer treatment[29].

Importantly, Boya *et al* have reported that when cells are inhibited at the final step of autophagy, which is the fusion of autophagosome and lysosome, cells die during type I PCD (apoptosis); on the other hand, when cells complete the whole process of autophagy, they survive. Thus, the inhibition of autophagy causes cells to undergo apoptosis, which suggests that there is cross-talk between type I and type II PCD[30]. Indeed, the results of annexin-V flow cytometry indicated that MK615 induced apoptosis after 6 h of incubation.

In conclusion, we demonstrated that an extract of compounds from Japanese apricot (*ume*), MK615, exhibits anti-proliferative effects against colon cancer cells *in vitro*, and induced massive autophagy in these cells. Although further study is needed, the natural compounds in MK615 appear to exert antineoplastic effects through induction of autophagy-related PCD in colon cancer cells.

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COMMENTS

Background

Japanese apricot, *Prunus mume* Sieb. et Zucc (*ume* in Japanese), has for centuries been a traditional Japanese medicine, and is a familiar and commonly consumed food. Some components of Japanese apricot have been shown to inhibit cancer cell growth.

Research frontiers

MK615 is an extract of compounds from Japanese apricot, and has been shown to possess an anti-proliferative effect against some cancer cell lines. Autophagy is a form of PCD and has been shown to regulate cancer-cell growth. However, there are not many substances that can stably induce autophagy in cancer cells.

Innovations and breakthroughs

In this report, we clearly described that MK615 inhibited the growth of colon cancer cells by aggressively inducing autophagy.

Applications

The side effects of chemotherapeutic agents often reduce the quality of life of patients with colorectal cancer. MK615 appears to be an effective and less toxic chemotherapeutic agent against colorectal cancer.

Terminology

Autophagy is classified as type Ⅱ PCD, and differs from type Ⅰ PCD (apoptosis). Autophagy is a form of caspase-independent cell death, displays no DNA-laddering, and is typically characterized by formation of cytoplasmic vacuoles. In recent years, the importance of autophagy has been stressed in various biological fields, including cancer.

Peer review

In this *in vitro* study, the authors investigated the antineoplastic effects of MK615, an extract from Japanese apricot (*ume* in Japanese), against colon cancer cells. They concluded that MK615 had an antineoplastic effect against colon cancer cells, and the effect may have been exerted by induction of apoptosis and autophagy.

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VIRAL HEPATITIS

Chronic hepatitis C virus infection: Prevalence of extrahepatic manifestations and association with cryoglobulinemia in Bulgarian patients

Diana V Stefanova-Petrova, Anelia H Tzvetanska, Elisaveta J Naumova, Anastasia P Mihailova, Evgenii A Hadjiev, Rumiana P Dikova, Mircho I Vukov, Konstantin G Tchernev

Diana V Stefanova-Petrova, Konstantin G Tchernev, Department of Internal Diseases and Gastroenterology, University Hospital Alexandrovska, Sofia 1431, Bulgaria
Anelia H Tzvetanska, Viral Laboratory, University Pediatric Hospital, Sofia 1431, Bulgaria
Elisaveta J Naumova, Anastasia P Mihailova, Central Laboratory of Immunology, University Hospital Alexandrovska, Sofia 1431, Bulgaria
Evgenii A Hadjiev, Department of Hematology, University Hospital Alexandrovska, Sofia 1431, Bulgaria
Rumiana P Dikova, Department of Pathology, University Hospital Alexandrovka, Sofia 1431, Bulgaria
Mircho I Vukov, National Centre for Health Information, Sofia 1431, Bulgaria
Correspondence to: Dr. Diana V Stefanova-Petrova, Department of Internal Diseases and Gastroenterology, University Hospital Alexandrovska, 1, Georgi Sofiiski Str, Sofia 1431, Bulgaria. d.petrova.stefanova@gmail.com
Telephone: +359-2-9478670 Fax: +359-2-9230654
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Abstract

AIM: To assess the prevalence of extrahepatic manifestations in Bulgarian patients with chronic hepatitis C virus (HCV) infection and identify the clinical and biological manifestations associated with cryoglobulinemia.

METHODS: The medical records of 136 chronically infected HCV patients were reviewed to assess the prevalence of extrahepatic manifestations. Association between cryoglobulin-positivity and other manifestations were identified using χ^2 and Fisher's exact test. Risk factors for the presence of extrahepatic manifestations were assessed by logistic regression analysis.

RESULTS: Seventy six percent (104/136) of the patients had at least one extrahepatic manifestation. Clinical manifestations included fatigue (59.6%), kidney impairment (25.0%), type 2 diabetes (22.8%), paresthesia (19.9%), arthralgia (18.4%), palpable purpura (17.6%), lymphadenopathy (16.2%), pulmonary fibrosis (15.4%), thyroid dysfunction (14.7%), Raynaud's phenomenon (11.8%), B-cell lymphoma (8.8%), sicca syndrome (6.6%), and lichen planus (5.9%). The biological manifestations included cryoglobulin production (37.5%), thrombocytopenia (31.6%), and autoantibodies: anti-nuclear (18.4%), anti-smooth muscle (16.9%), anti-neutrophil cytoplasm (13.2%) and anti-cardiolipin (8.8%). All extrahepatic manifestations showed an association with cryoglobulin-positivity, with the exception of thyroid dysfunction, sicca syndrome, and lichen planus. Risks factors for the presence of extrahepatic manifestations (univariate analysis) were: age $\geqslant$ 60 years, female gender, virus transmission by blood transfusions, longstanding infection ($\geqslant$ 20 years), and extensive liver fibrosis. The most significant risks factors (multivariate analysis) were longstanding infection and extensive liver fibrosis.

CONCLUSION: We observed a high prevalence of extrahepatic manifestations in patients with chronic HCV infection. Most of these manifestations were associated with impaired lymphoproliferation and cryoglobulin production. Longstanding infection and extensive liver fibrosis were significant risk factors for the presence of extrahepatic manifestations in HCV patients.

Key words: Hepatitis C; Liver cirrhosis; Extrahepatic manifestations

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INTRODUCTION

Hepatitis C virus (HCV) is associated with a wide spectrum of clinical and biological extrahepatic manifestations[1-3]. In chronically infected patients, the virus can trigger an impairment in lymphoproliferation with cryoglobulin production[3]. Mixed cryoglobulinemia with its complications (skin, neurological, renal, and rheumatologic) is the most significant extrahepatic manifestation of HCV

infection[4,5]. Mixed cryoglobulinemia can evolve into B-cell lymphoma in up to 10% of the patien[6,7].

Various non-organ specific auto-antibodies, present in low titers, are noted during the course of chronic HCV infection[8,9], but they do not influence the clinical profile of the disease[10]. Chronic HCV infection has been linked to two skin disorders, porphyria cutanea tarda and lichen planus, particularly with the involvement of the oral cavity[11,12]. Other possible HCV-associated diseases are type 2 diabetes mellitus[13], thrombocytopenia[14] and pulmonary fibrosis[15]. The sicca syndrome, although different from the typical Sjogren's syndrome, also appears to be associated with hepatitis C virus infection[16,17].

It is unclear whether HCV plays a pathogenic role in the development of thyroid dysfunction[18]. It is possible, that this extrahepatic manifestation of HCV is related to treatment with interferon rather than the virus[19].

The most common risk factors associated with extrahepatic manifestations of HCV infection are older age, female sex, and extensive liver fibrosis[8]. Several reports from different parts of the world suggest that hepatitis C virus affects not only the liver, but other tissues, organs and systems as well. Lymphoproliferative disorders, triggered by the virus, are the most significant extrahepatic manifestations in South-Eastern Europe, where Bulgaria is located.

The aim of the present study was to determine the prevalence of various extrahepatic manifestations of chronic HCV infection in our country, to analyze which extrahepatic manifestations are associated with impaired lymphoproliferation and cryoglobulin production, and to identify which patients are at greater risk of developing extrahepatic manifestations.

In our clinical practice, several patients present with extrahepatic manifestations even in the absence of a clearly defined clinical picture of hepatic illness. It is important to recognize these manifestations in order to make an early diagnosis and to initiate therapy in a timely manner.

MATERIALS AND METHODS

Patients

We included 136 Bulgarian patients who were referred to the Department of Internal Diseases and Gastroenterology of the University Hospital Alexandrovska during the period from 1996 to 2004. The main reason for patient referral was elevated liver enzymes. The diagnosis of HCV infection was made by the presence of anti-HCV antibodies (third generation ELISA) and a positive test for HCV-RNA (Cobas Amplicor HCV Monitor Test, v. 2.0, Roche Diagnostics). HCV genotypes were identified by direct sequence analysis (Immunogenetics, Belgium) in 60 patients[20]. HBsAg and HIV positive patients were not included in the study. Liver biopsy was performed in all patients. The data was collected before the patients were started on any specific treatment.

Methods

Patient records were reviewed to assess the presence of the following clinical manifestations: fatigue, arthralgia, Raynaud's phenomenon, palpable purpura, paresthesia, renal impairment (proteinuria, creatinine above the upper normal limit, and hypertension), sicca syndrome (mouth and eyes), thyroid dysfunction (TSH level below or above the normal range of 0.31-5.00 mU/L), lichen planus (skin and oral lesions), type 2 diabetes mellitus (hyperglycemia, treated by hypoglycemic drugs), pulmonary fibrosis (X-ray examination), lymphadenopathy, and lymphoma. The enlargement of peripheral lymph nodes was detected by palpation. The enlargement of mediastinal lymph nodes was detected by X-ray examination, and abdominal lymphadenopathy was detected by abdominal ultrasonography. Chest X-ray and abdominal ultrasonography were obligatory exams carried out routinely in all patients admitted to the hospital. Lymph node enlargement was confirmed by computerized tomography. The diagnosis of lymphoma was based on morphologic evaluation of lymph node tissue in the patients with co-existing peripheral lymphadenopathy, and by bone marrow biopsy. Histological analysis of the enlarged mediastinal and/or abdominal lymph nodes was not performed because patients refused to provide informed consent for invasive diagnostic surgical procedures.

Biological data obtained from for each patient included the presence or absence of cryoglobulins, thrombocytopenia, and auto-antibodies: anti-nuclear (ANA), anti-smooth muscle (ASMA), anti-neutrophil cytoplasm (ANCA), and anti-cardiolipin (ACL). The platelet count was obtained from the patients' files. Thrombocytopenia was defined as platelet count $\leqslant 110.10^9$/L. Cryoglobulins were detected by the Winfield method[21]. Twenty milliliters of venous blood was obtained from each patient in a pre-warmed (37℃) syringe, allowed to clot at 37℃ and the serum was separated by centrifugation. The supernatant was incubated at 4℃ for 8 d and examined daily for cryoprecipitate. Indirect immunofluorescence was used for the detection of ANA, ASMA, and ANCA (IFA/ Binding Site). A positive test for ANA and ASMA was defined as a titer $\geqslant$ 1/40, and for ANCA $\geqslant$ 1/20. Anticardiolipin antibodies (ACL-IgG and ACL-IgM) were detected by ELISA (Orgentec).

The following demographic and epidemiologic data was collected: age (less or $\geqslant$ 60 years), sex, the suspected duration of the infection (less or $\geqslant$ 20 years), an alcohol intake (less or $\geqslant$ 50 g/d), and the mode of infection. Questions regarding the most relevant identifiable HCV risks factors were asked, including transfusion of blood and blood products, intravenous drug use, surgical procedures, dental manipulation associated with bleeding, and needle stick injury in health workers. In patients with a past history of transfusions of blood and blood products, it was assumed that HCV infection was caused by contaminated blood and blood products.

The histological abnormalities in liver biopsy specimens, obtained blindly (Hepafix 1.4 mm, B. Braun, Germany), were scored according to the METAVIR system[22]. Each liver biopsy sample was assessed for the stage of fibrosis and grade of histological activity. Liver fibrosis was staged on a scale of 0 - 4, where 0 = no fibrosis, 1 - portal fibrosis without septa, 2 = few septa,

Table 1 Prevalence of clinical and biological extrahepatic manifestations in 136 patients with chronic HCV infection

Clinical manifestations	*n* (%)
Fatigue	81 (59.6)
Renal involvement	34 (25.0)
Type 2 diabetes mellitus	31 (22.8)
Paresthesia	27 (19.9)
Arthralgia	25 (18.4)
Purpura	24 (17.6)
Pulmonary fibrosis	21 (15.4)
Thyroid dysfunction	20 (14.7)
Raynaud's phenomenon	16 (11.8)
B-cell lymphoma	12 (8.8)
Sicca-syndrome (xerostomia)	9 (6.6)
Lichen planus	8 (5.9)
Biological manifestations	
Cryoglobulinemia	51 (37.5)
Thrombocytopenia	4 (31.6)
ANA	25 (18.4)
ASMA	23 (16.9)
ANCA	18 (13.2)
Anticardiolipin antibodies	12 (8.8)
Overall	
At least 1 extrahepatic manifestation	104 (76.5)

ANA: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; ANCA: Anti-neutrophil cytoplasm antibodies.

3 = numerous septa without cirrhosis, and 4 = cirrhosis. Necroinflammatory activity was graded on a scale of A0-A3, where A0 = no histological activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity.

Statistical analysis

Quantitative data was expressed as mean ± SD. Univariate analysis used the χ^2- square or Fisher's exact test for comparison of qualitative values. An assessment of the characteristics of HCV infection (demographic, epidemiologic, histologic), associated with the presence of extrahepatic manifestations, was performed using univariate and multivariate logistic regression analysis. Statistical significance was assessed at $P < 0.05$. Adjusted odds ratio (OR) and 95% confidence intervals (CI) were derived from the coefficient of the final multivariate logistic model. The analysis was performed with SPSS v.12.0 statistical software.

RESULTS

A total of 136 patients with chronic HCV infection were included in the study, comprising of sixty-two (45.6%) males and seventy-four (54.4%) females. The mean age of the patients was 50.16 ± 16.08 years (range 20-80 years). Forty-six patients (33.8%) were ⩾ 60 years of age. Thirty-four patients (25.0%) had a history of alcohol intake (⩾ 50 g/d). The suspected duration of infection was ⩾ 20 years in 70 patients (51.5%) and < 20 years in 66 patients (48.5%). Genotyping was performed in sixty patients, fifty-two (87%) had genotype 1, 4 (6.5%) genotype 3, and 4 (6.5%) had mixed genotypes. Metavir scores for inflammatory activity were: A0 in 5 patients (3.7%), A1 48 (35.3%), A2 46 (33.8%), and A3 37 patients (27.2%). Metavir scores for fibrosis were: F0 in 12 patients (8.8%), F1 16 (11.8%), F2 25 (18.4%), F3 27 (19.9%), and F4 in 56 patients (41.1%). Twenty patients (14.7%) were intravenous drug users. Thirty-seven patients (27.2%) were possibly infected during surgical procedures. History of dental manipulation with bleeding was detected in 18 patients (13.2%). Needle stick injury was found in 11 health workers (8.1%). Fifty of the 136 patients (36.8%) had previously been transfused with blood or blood products. Thirty-eight of these 50 patients were women, who received blood transfusions during childbirth, and in 33 of these patients the duration of infection was ⩾ 20 years. Nineteen of these 38 women were ⩾ 60 years of age at the time of presentation. Twenty of these patients had Metavir stage F4, and 10 (10/38) were Metavir F3.

The overall prevalence of individual extrahepatic manifestations is shown in Table 1. At least one extrahepatic manifestation was identified in 104 patients (76.5%). The clinical manifestations, in descending order of prevalence were: fatigue (59.6%), kidney impairment (25.0%), type 2 diabetes mellitus (22.8%), paresthesia (19.9%), arthralgia (18.4%), palpable purpura predominantly of the lower extremities (17.6%), lymphadenopathy (16.2%), pulmonary fibrosis (15.4%), thyroid dysfunction (14.7%), Raynaud's phenomenon (11.8%), B-cell non-Hodgkin's lymphoma (8.8%), sicca-syndrome (6.6%), and lichen planus (5.9%). All patients with sicca-syndrome had xerostomia, but none had xerophtalmia. Three patients had skin lesions of lichen planus and five had oral lesions. The biological manifestations, in descending order of prevalence were: cryoglobulin (37.5%), thrombocytopenia (31.6%), ANA (18.4%), ASMA (16.9%), ANCA (13.2%), anti-cardiolipin antibodies (8.8%). The ANA, ASMA and ANCA were present in low titers (⩽ 1/160). None of the patients in the study had clearly defined clinical features of autoimmune liver disease.

The relationship between the presence of cryoglobulins and other extrahepatic manifestations is shown in Table 2. Cryoglobulin-positivity was related to the following clinical manifestations: fatigue, purpura, Raynaud's phenomenon, kidney impairment, type 2 diabetes mellitus, arthralgia, paresthesia, pulmonary fibrosis, lymphadenopathy, and B-cell lymphoma. Cryoglobulin-positivity was also associated with the following biological manifestations: thrombocytopenia, and positive ANA, ASMA, ANCA, and anti-cardiolipin autoantibodies. Three female patients (⩾ 60 years at the time of the study) with advanced liver fibrosis and history of blood transfusions during childbirth, first became cryoglobulin positive and subsequently developed lymphadenooathy over two, three, and five years respectively. Histological analysis of the removed lymph nodes showed low-grade non Hodgkin B-cell lymphoma. The present study did not show any association between the presence of cryoglobulins and thyroid dysfunction, sicca syndrome, and lichen planus (Table 2).

The demographic, epidemiological and liver histological features associated with the extrahepatic manifestations were analyzed by univariate and multivariate logistic regression analysis. The results are shown in Table 3. Using univariate logistic regression analysis there was a positive correlation

Table 2 Relationship between cryoglobulin-positivity and the presence (%) of extrahepatic manifestations in patients with chronic HCV infection

Extrahepatic manifestations	Cryoglobulin-positive patients (n = 51)	Cryoglobulin-negative patients (n = 85)	P	Odds ratio (95% confidence interval)
Fatigue	92.2	40.0	0.000	17.6 (5.8-53.4)
Purpura	41.2	3.5	0.000	19.1 (5.3-68.8)
Raynaud' phenomenon	25.5	3.5	0.000	9.3 (2.5-34.7)
Arthralgia	33.3	9.4	0.001	4.8 (1.8-12.2)
Paresthesia	41.2	7.1	0.000	9.2 (3.3-25.0)
Renal involvement	45.1	12.4	0.000	5.5 (2.3-12.7)
Type 2 diabetes	35.3	15.3	0.011	3.0 (1.3-6.8)
Lymphadenomegaly	35.3	4.7	0.000	11.0 (3.4-35.1)
Lymphoma	17.6	3.5	0.015	5.8 (1.5-22.7)
Pulmonary fibrosis	25.5	9.4	0.015	3.2 (1.2-8.6)
Thyroid dysfunction	15.7	14.1		
Xerostomia	11.8	3.5		
Lichen planus	7.8	4.7		
Thrombocytopenia	58.8	15.3	0.000	7.9 (3.5-17.8)
ANA	29.4	11.8	0.013	3.1 (1.2-76.0)
ASMA	33.3	7.1	0.000	6.5 (2.3-18.1)
ANCA	25.5	5.9	0.002	5.4 (1.9-16.4)
ACL-antibodies	17.6	3.5	0.010	5.8 (1.5-22.7)

ANA: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; ANCA: Anti-neutrophil cytoplasm antibodies; ACL: Anti-cardiolipin.

Table 3 Univariate and multivariate logistic regression analysis of factors associated with the presence of extrahepatic manifestations in patients with chronic HCV infection

Factors	Patients with the factor (%) in the presence of extrahepatic manifestations (n = 104)	Patients with the factor (%) in the absence of extrahepatic manifestations (n = 32)	Uni-variate analysis	Multi-variate analysis	Odds ratio (95% confidence interval)
Female sex	59.6	37.5	0.042		
Age ⩾ 60 yr	43.3	3.1	0.000		
Duration of infection ⩾ 20 yr	66.3	3.1	0.000	0.001	36.3 (4.6-284.9)
Transfusion of blood and blood products	43.3	15.6	0.006		
Metavir A3	30.8	15.6			
Metavir F4	52.9	3.1	0.000	0.011	15.4 (1.8-125.9)
Alcohol ⩾ 50 g/d	24.0	28.1			

between the presence of extrahepatic manifestations and female sex, older age (⩾ 60 years), duration of the infection (⩾ 20 years), transfusion of blood and blood products, and extensive liver fibrosis (Metavir F4). Univariate analysis did not show any correlation between extrahepatic manifestations and the mode of transmission: intravenous drug use (8.7% *vs* 34.4% for the patients without extrahepatic manifestations), surgical procedures (27.9% *vs* 25.5%), dental manipulation with bleeding (12.5% *vs* 15.6%), and needle stick injury in health workers (7.7% *vs* 9.4%). Univariate logistic regression analysis did not show an association between extrahepatic manifestations and high grade of inflammatory activity in the liver (Metavir A3) and alcohol intake of ⩾ 50 g/d (Table 3). Multivariate logistic regression analysis showed that the most significant association was between extrahepatic manifestations and long duration of infection and advanced liver fibrosis (Table 3).

DISCUSSION

The present study on 136 patients with chronic HCV infection, showed a high prevalence of extrahepatic clinical and biological manifestations. At least one manifestation was present in 76.5% of the patients. These results are similar to those reported in a large prospective French study, in which 74% of 1614 patients with chronic HCV infection had at least one extrahepatic clinical symptom[23]. By univariate analysis (Table 3) five risks factors were found to be related with clinical and biological extrahepatic manifestations in our patients: female sex, age ⩾ 60 years, transfusion of blood and blood products, duration of the infection ⩾ 20 years, and extensive liver fibrosis. By multivariate analysis (Table 3) the most significant risks factors were the longstanding infection and extensive liver fibrosis.

Fatigue was the most frequent non-specific clinical symptom in chronic HCV infection[2]. About 60% patients in this study considered fatigue as the initial or worst symptom of their disease (Table 1). In a prospective study of 1614 individuals with chronic HCV infection, fatigue was present in 53% of patients[24]. It is not known what causes fatigue in chronic HCV infection. The elevated fatigue score[25] is probably related to an increase in serum leptin levels which may interact with serotonin neurotransmission[26]. Fatigue can be considered a part of

the clinical picture of HCV-associated cryoglobulinemia, since this symptom was seen more frequently in cryoglobulin-positive than in cryoglobulin-negative patients (92.2% *vs* 40.0%, OR 17.6, 95% CI 5.8-53.4).

Hepatitis C virus escapes immune elimination in chronically infected patients[5]. In such patients, CD81-mediated activation of B cells triggers mono-oligoclonal B-lymphocyte expansion and the appearance of various HCV-related autoimmune disorders, including the syndrome of mixed cryoglobulinemia[6,27]. A recent meta-analysis showed that 44% patients with chronic HCV infection had circulating immune complexes with cryoprecipitating properties[28]. In the present study, cryoglobulins were isolated in 51 of the 136 (37.5%) patients, but we were not able to define the type of cryoglobulin in all the cases. However, there was considerable evidence to suggest that HCV was associated with type Ⅱ mixed cryoglobulinemia, with clinical features of vasculitis which mainly affects the small sized blood vessels of the skin, joints, nerves, and kidneys[5,29]. Skin is the most frequently involved target organ[30]. In previous studies, palpable purpura was observed in 10% to 21% patients with clinically manifested cryoglobulinemic syndrome[11], and in 7% of all HCV infected patients[8]. We observed palpable purpura, predominantly over the lower extremities, in 24 of the 136 patients (17.6%), with much higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (41.2% *vs* 3.5%, OR 19.1, 95% CI 5.3-68.8). Symptoms related to Raynaud' s phenomenon were observed in 16 of the 136 patients (11.8 %), with higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (25.5% *vs* 3.5%, OR 9.3, 95% CI 2.5-34.7). Pruritus, Raynaud's phenomenon, and palpable purpura of the lower extremities are the main skin manifestations of chronic HCV infection, which is consistent with the findings of previous studies[11,31,32]. However, considering the high frequency of cryoglobulins in HCV patients, severe symptomatic mixed cryoglobulinemia with the clinical presentation of diffuse vasculitis was rare, seen in only 1% of cryoglobulin-positive patients[23]. Polyarteritis nodosa-type of clinical presentation was not observed in the present study.

Previous studies have shown that arthralgia is present in 44.7% patients with HCV-associated mixed cryoglobulinemia[33] and in 19% of all HCV infected patients[8]. The clinical picture may mimic rheumatoid arthritis, especially since rheumatoid factor was present in 71% of cases[34], but there was no joint destruction, and antibodies to cyclic citrullinated peptides, which are highly specific for rheumatoid arthritis, were absent[35]. In the present study, arthralgia was present in 18.4% patients, with higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (33.3% *vs* 9.9%, OR 4.8, 95% CI 1.8-12.2). None of the patients in the present study met the diagnostic criteria for rheumatoid arthritis.

Peripheral nervous system involvement is a characteristic feature of the more severe forms of clinically apparent HCV-cryoglobulinemia[3]. Peripheral neuropathy, manifested by paresthesia, was noted in 17% of all HCV patients[23] and in 37% to 80% of the patients with HCV-associated mixed cryoglobulinemia[36,37]. We observed paresthesia in 19.9% of all HCV infected patients, with a higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (41.2% *vs* 7.1%, OR 9.2, 95% CI 3.3-25.0). Sensory nerves are mainly affected in patients with HCV-associated mixed cryoglobulinemia[38]. Neuropathological data shows axonal degeneration, differential fascicular loss of axons, signs of demyelinization, and small-vessel vasculitis with mononuclear cell infiltrates in the perivascular areas[38,39].

Chronic HCV infection can trigger the immune complex syndrome of cryoglobulin deposition and type-1 membranoproliferative glomerulonephritis[40]. Diffuse membranoproliferative glomerulonephritis is found in 83% of cryoglobulinemic renal disease[41]. Misiani *et al*[42] found a high prevalence of HCV antibodies (66%) and HCV RNA (81%) in the serum of patients with cryoglobulinemic glomerulonephritis. Only 2% of the controls (patients with noncryoglobulinemic glomerulopathies) had HCV antibodies[42]. In Japan, the virus was found in 60% patients with membranoproliferative glomerulonephritis[43]. Clinically obvious renal disease was present in 20% to 30% of cryoglobulin-positive patients with HCV infection[7,23,44]. In 55% of these patients, the findings include mild proteinuria, mild microscopic hematuria and mild renal insuffiency[45,46]. Arterial hypertension is present in 80% patients[45,47]. In the present study, 34 of the 136 HCV patents (25%) had mild proteinuria, hypertension and serum creatinine levels above the upper limit of normal. These symptoms are related to HCV-cryoglobulinemia, since their prevalence was higher in cryoglobulin-positive than in cryoglobulin-negative patients (45.1% *vs* 12.4%, OR 5.5, 95% CI 2.3-12.7). Renal abnormalities during the course of HCV infection are usually diagnosed in most patients between the fifth and sixth decades of life, and occur slightly more frequently in women than men[45,48]. Risk factors for the development of severe renal failure at follow-up of these patients include age, serum creatinine level, and proteinuria at the onset of renal disease[41]. Cryoglobulin-related nephropathy has been reported to progress to chronic renal failure requiring dialysis in 10% patients[44], but the overall survival at 10 years was 80%[41].

Case-control studies show an increase in the prevalence of type 2 diabetes mellitus (14.5% to 24%) in patients with chronic HCV infection[13,49,50]. These findings have been confirmed in a representative sample of the general population in the USA[51]. Reports from diverse geographic regions have shown a 2- to 10- fold increase in the prevalence of diabetes in patients with HCV infection compared to liver disease controls[51-53]. The highest prevalence (up to 50%) was noted in patients with HCV-associated liver cirrhosis[54-56]. Our findings are consistent with these studies. We found type 2 diabetes in 31 of the 136 HCV patients (22.8%), which is higher than the prevalence (8.0%) in the general population of our country. Antonelli *et al*[57] found a higher prevalence (14.4%) of type 2 diabetes in HCV patients with mixed cryoglobulinemia compared to HCV-negative age-matched controls (6.9%) and the general population in northern Italy (2.5%-3.3%). We also found a higher prevalence of type 2 diabetes in cryoglobulin-positive than in cryoglobulin-negative patients (35.3% *vs* 15.3%, OR 3.0,

95% CI 1.3-6.8). Given the biology of HCV, which is both hepatotropic and lymphotropic, an immune-mediated mechanism may explain the raised prevalence of type 2 diabetes in HCV-patients with mixed cryoglobulinemia[57]. Recent studies suggest that insulin resistance mediated by proinflammatory cytokines, rather than a deficit in insulin secretion, is the primary pathogenic mechanism involved in the development of type 2 diabetes in HCV infection[58].

Soresi *et al*[59] detected abdominal lymphadenopathy in 22% patients with HCV infection and persistently normal transaminases, and in 38% of those with high alanine aminotransferase values. Multiple logistic regression analysis showed a significant relationship between abdominal lymphadenopathy and histological abnormalities of the liver, presence of HCV RNA in the serum and gamma-globulin levels[59]. Lymphadenopathy in chronic HCV infection indicates a possible interaction between viral antigens and the immune system[60]. This interaction may be complicated by autoimmunity, cryoglobulinemia and B cell malignancy[60]. In the present study, lymphadenopathy was seen in 22 of the 136 HCV patients (16.2%), with a higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (35.3% *vs* 4.7%, OR 11.0, 95% CI 3.4-35.1). Epidemiological studies have confirmed a link between HCV infection and B-cell non Hodgkin's lymphoma[61-63]. In a recent meta-analysis, the prevalence of HCV infection in patients with B-cell non Hodgkin's lymphoma was approximately 15%, which is much higher than the prevalence of HCV in the general population[64]. Clonal B cell proliferation was observed in patients with a longer duration of HCV infection, type Ⅱ cryoglobulin, and vasculitis[5,65,66]. Over 90% patients who developed non-Hodgkin lymphoma had type Ⅱ cryoglobulins[64]. About 10% patients with HCV mixed cryoglobulinemia evolved into lymphoma[6]. In a series of 231 Italian patients with mixed cryoglobulinemia, 20 developed B-cell lymphoma after a mean of 8.8 years[7]. The overall risk of non-Hodgkin lymphoma in patients with cryoglobulinemic syndrome was 35 times higher than that in the general population[64]. Our findings are in agreement with the results reported from Italy[67]. In the present study, B-cell non Hodgkin's lymphoma was diagnosed in 12 of the 136 HCV patients (8.8%) with higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (17.6% *vs* 3.5%, OR 5.8, 95% CI 1.5-22.7). The exact prevalence of HCV associated lymphoma in our country may be much higher, since patients with abdominal and/or mediastinal lymphadenopathy (without enlargement of peripheral lymph nodes) did not provide consent for invasive diagnostic surgical procedures. The appearance of cryoglobulins, peripheral lymphadenopathy and low-grade B-cell lymphoma in 3 women during follow-up suggested that benign lymphoproliferation, triggered by the virus, can evolve into malignant B cell lymphoma. It is possible that genetic and environmental factors[68] play a role in the higher prevalence of HCV-associated lymphoma in South-East Europe, including our country, compared to Northern Europe[69]. Based on the available evidence it appears that HCV is an important risk factor for B-cell malignancy in areas with a high prevalence of HCV infection[5], which in our country is 1.3%.

Ueda *et al*[70] noted a higher prevalence of anti-HCV antibodies in Japanese patients with idiopathic pulmonary fibrosis compared to the general population. The prevalence of idiopathic pulmonary fibrosis in anti-HCV positive patients was 9.4%[71]. HCV patients with mixed cryoglobulinemia have been found to have asymptomatic interstitial lung fibrosis diagnosed by chest X-ray and high-resolution computed tomography[15]. Pulmonary fibrosis in HCV-related cryoglobulinemia is perhaps triggered by local deposition of circulating HCV-containing immune complexes[72]. In the present study 21 of the 136 HCV patients (15.4%) showed pulmonary fibrosis on routine X-ray examination; the frequency of pulmonary fibrosis was higher in cryoglobulin-positive than in the cryoglobulin-negative patients (25.5% *vs* 9.4%, OR 3.2, 95% CI 1.2-8.6). These findings need further confirmation with the use of more sophisticated radiological techniques.

Serum autoantibodies are commonly seen in patients with chronic HCV infection. In a study by Lenzi *et al*[9] the overall prevalence of non-organ specific autoantibodies was significantly higher in anti-HCV positive patients compared to healthy subjects, and HBsAg positive controls (25% *vs* 6% and 7% respectively). Several studies have evaluated the prevalence of ANA and ASMA, with figures ranging from 4.4% to 41%[8,10,34] and 7% to 66% respectively[23,34,73]. In the present study, positive ANA and ASMA tests were seen in 18.4% and 16.9% patients respectively. The anti-ANA and ASMA antibodies were found in low titers ($\leqslant$ 1/160), and were detected predominantly in the cryoglobulin-positive patients (Table 2). However, none of the patients had a clearly defined clinical picture of autoimmune liver disease. Recent data suggests that the HCV core particles concentrated in the cryoprecipitate play a role in the interaction between the cryoglobulins, endothelial cells and neutrophil granulocytes[74]. The main antigens in HCV patients with a positive ANCA test are proteinase 3 and dihydrolipoamine dehydrogenase[75]. A positive ANCA has been found in 10% patients with HCV-associated mixed cryoglobulinemia[76]. In the present study, ANCA was present in 18 of the 136 patients (13.2%), with higher prevalence in cryoglobulin-positive than cryoglobulin-negative patients (25.5% *vs* 5.9%, OR 5.4, 95% CI 1.9-16.4). HCV patients with ANCA had a higher prevalence of skin involvement, anemia, abnormal liver function tests and elevated alpha feto-protein levels[75]. Anticardiolipin antibodies have been observed in 3.3% to 22% of HCV patients[77-79]. These antibodies were found more frequently in patients with HCV-associated cryoglobulinemia[80]. Our findings are consistent with previously reported data. In the present study, 12 of the 136 patients (8.8%) had anticardiolipin antibodies, with higher prevalence in cryoglobulin-positive than in cryoglobulin-negative patients (17.6% *vs* 3.5%, OR 5.8, 95% CI 1.5-22.7). According to a recent study, these antibodies do not have any clinical significance in HCV patients, since they are anti-β2-glicoprotein I independent[78]. The auto-antibodies seen in patients with HCV infection appear to resemble those seen in chronic viral infections - the autoantibody titers are low, frequently found during the clinical course

of cryoglobulinemia, but do not produce a typical autoimmune disease[29,80].

The prevalence of thrombocytopenia is higher in patients with chronic HCV infection than in the general population[14]. In a series of 368 patients with chronic HCV infection, Nagamine *et al*[81] detected 151 cases (41%) of thrombocytopenia. Our findings also suggest a high prevalence of thrombocytopenia in patients with chronic HCV infection (31.6%). This can be explained by the large number of patients with cirrhosis in the present study. Platelet sequestration and destruction in the spleen[82,83], along with low thrombopoetin production[83,84] play an important role in the pathogenesis of thrombocytopenia. However, a direct viral infection of the megakariocytes[85] and autoimmune mechanisms[86,87] cannot be ruled out. The higher prevalence of thrombocytopenia in cryoglobulin-positive than in cryoglobulin-negative patients (58.8% *vs* 15.3%, OR 7.9, 95% CI 3.5-17.8) in the present study may reflect the role of immune dysregulation in causing both abnormalities.

In previous studies, thyroid specific antibodies were observed in 22.1% patients with chronic HCV infection, but the presence of thyroid dysfunction was not different compared to age and gender matched controls[18]. Thyroid dysfunction was seen in 14.7% of our 136 untreated HCV patients, and there was no association with the presence of cryoglobulins. However, up to 12.6% HCV patients developed thyroid dysfunction during or after IFN therapy[88-90], with a significant association with female gender[91].

Lymphocytic infiltration of the salivary glands has been described in 57% patients with chronic HCV infection compared to 5% in deceased controls[16]. A direct role of the virus is suggested by studies on mice expressing HCV envelope proteins E1 and E2 in the liver and salivary glands[92]. Sialadenitis developed in 84% HCV infected mice compared to 2% in control littermates and 0% in HCV core transgenic animals[92]. Up to 10% patients with chronic HCV infection may develop symptoms of xerostomia and xerophthalmia[8], whereas less than 5% of patients with confirmed Sjogren syndrome are carriers of HCV[17]. In the present study, xerostomia was seen in 6.6% patients, but none had xerophthalmia. Our results show a much higher prevalence of xerostomia in cryoglobulin-positive patients, however there was no difference compared to those without cryoglobulins (Table 2). In a French study, the classic Sjogren syndrome (xerostomia, xerophthalmia, histological stages Ⅲ and Ⅳ in the Chilshom scale, and anti-SSA or anti-SSB antibodies) was observed only in 1% of patients with chronic HCV infection[32].

Clinical studies, predominantly from the Mediterranean area and Japan, suggest that lichen planus, mainly the oral form, is associated with HCV infection[93-95]. The overall prevalence of anti-HCV antibodies in Spanish patients with lichen planus was 29.2%[12]. Nagao *et al*[93] found serum anti-HCV antibodies in 28 (62%) and serum HCV RNA in 27 (60%) of 45 Japanese patients with oral lichen planus. However, these results have not been confirmed by reports from other regions[32,96]. The geographical differences could be related to immunogenetic factors since the allele HLA-DR6 is expressed in a significant proportion of Italian patients with oral lichen planus and HCV infection[95]. The frequency of lichen planus in our HCV patients (5.9%) is similar to that reported by Pawlotsky *et al*[32] in France (5%). We found oral lesions of lichen planus in 5 patients and skin lesions in 3 patients. In the present study, we did not find any association between cryoglobulinemia and the presence of lichen planus (Table 2). Pilli *et al*[97] detected HCV-specific T cells with phenotypic and functional characteristics of terminally differentiated effector cells at the site of the oral lesions. These findings and the detection of HCV RNA strands in the lichen tissue[98] suggests a possible role for HCV-specific T-cell responses in the pathogenesis of lichen planus associated with HCV infection[99].

Our study has demonstrated a high prevalence of extrahepatic manifestations in Bulgarian patients with chronic HCV infection. Most of these manifestations are the consequence of impaired lymphoproliferation and show an association with cryoglobulin production. Clinical features such as fatigue, palpable purpura, Raynaud's phenomenon, arthralgia, paresthesia, kidney impairment, type 2 diabetes, pulmonary fibrosis, lymphadenopathy, B cell lymphoma, thrombocytopenia and non-organ specific autoantibodies can be regarded as a part of the clinical spectrum of HCV-associated cryoglobulinemia. On the other hand, thyroid dysfunction, sicca-syndrome and lichen planus are also seen in patients with chronic HCV infection, but were not associated with the presence of cryoglobulins in the present study. Extrahepatic manifestations were observed more frequently in older women with longstanding infection, advanced liver fibrosis and HCV infection acquired through transfusion of blood and blood products during childbirth. These findings suggest that the dose of the virus and the route of transmission may be important factors associated with extrahepatic manifestations. The long duration of HCV infection and extensive liver fibrosis were the most frequent findings associated with extrahepatic manifestations, but the pathogenesis of such an association is unclear[1,23]. Progression of liver fibrosis and the development of systemic-portal venous anastomosis allows the passage of antigens directly into the systemic circulation, thus bypassing the liver filter and resulting in chronic interaction with the immune system[23]. Some of the immunological defects are the consequence of HCV interaction with dendritic cells and a shift from Th1 to Th2 cytokine profile[100,101].

Patients with chronic HCV infection have a high prevalence of extrahepatic manifestations. These manifestations are found more frequently in the patients with a long duration of infection and liver cirrhosis, even in the absence of a clearly defined clinical picture of hepatic illness. Most of these manifestations are associated with impaired lymphoproliferation and cryoglobulin production, triggered by hepatitis C virus. The benign lymphoproliferation, associated with cryoglobulinemia, can evolve into malignant B cell lymphoma. Physicians should be aware of the extrahepatic signs and symptoms of HCV infection. HCV should be tested in all patients with these manifestations. This may lead to early diagnosis and successful treatment of chronic HCV infection.

COMMENTS

Background

Hepatitis C virus (HCV) affects not only the liver, but other tissues, organs, and systems as well. In our practice, several patients with chronic HCV infection present with extrahepatic manifestations, even in the absence of a clearly defined clinical picture of hepatic illness. Lymphoproliferative disorders, triggered by the virus, are frequently seen in South-Eastern Europe, where Bulgaria is located.

Research frontiers

The aim of the present study was to assess the prevalence of different extrahepatic manifestations in Bulgarian patients with chronic HCV infection, to identify extrahepatic manifestations associated with impaired lymphoproliferation and cryoglobulin production, and to determine which patients are at greater risk of developing extrahepatic manifestations of chronic HCV infection.

Innovations and breakthroughs

We observed a high prevalence of extrahepatic manifestations (76.5%) in patients with chronic HCV infection. Patients with longstanding infection and cirrhosis had a higher risk of developing such manifestations. In the present study, most of the extrahepatic manifestations were associated with impaired lymphoproliferation and cryoglobulin production, with the exception of sicca syndrome, thyroid dysfunction and lichen planus. The presence of B-cell non Hodgkin's lymphoma was observed in 8.8% patients, with a higher prevalence in cryoglobulin-positive compared with cryoglobulin-negative patients (17.6% *vs* 3.5%, OR 5.8, 95% CI 1.5-22.7).

Applications

Physicians should be aware of the extrahepatic signs and symptoms of HCV infection. HCV should be tested in patients who have any of these manifestations. This may lead to early diagnosis and successful treatment of chronic HCV infection. The treatment of lymphoproliferative disorders, including B-cell non Hodgkin's lymphoma poses a challenge, and requires new therapeutic strategies.

Terminology

Hepatitis C virus (HCV) escapes immune elimination. HCV interacts with hepatocytes and B lymphocytes through a common (CD81) receptor. CD81-mediated activation of B cells in chronically infected patients can trigger clonal B-lymphocyte proliferation with cryoglobulin' production, and evolution of disease into B-cell non Hodgkin's lymphoma. HCV can interact, with or without immune mediated mechanisms, with different tissues, organs, and systems.

Peer review

The authors assessed the prevalence of extrahepatic manifestations in Bulgarian patients with chronic HCV infection and identified the clinical and biological manifestations associated with cryoglobulinemia.

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BASIC RESEARCH

Calcitriol analog ZK191784 ameliorates acute and chronic dextran sodium sulfate-induced colitis by modulation of intestinal dendritic cell numbers and phenotype

Ulrike G Strauch, Florian Obermeier, Nicole Grunwald, Nadja Dunger, Heiko C Rath, Jürgen Schölmerich, A Steinmeyer, U Zügel, HH Herfarth

Ulrike G Strauch, Florian Obermeier, Nicole Grunwald, Nadja Dunger, Heiko C Rath, Jürgen Schölmerich, HH Herfarth, Department of Internal Medicine I , University of Regensburg, Regensburg D-93053, Germany
A Steinmeyer, Medicinal Chemistry, Schering AG, Berlin, Germany
U Zügel, Research Business Area Dermatology, Schering AG, Berlin, Germany
Supported by Grants from the German Research Foundation DFG (UGS, HCR), as well as research grants from the University of Regensburg, Germany, as part of the ReForM-program (UGS, FO)
Correspondence to: Ulrike G Strauch, Department of Internal Medicine I , University of Regensburg, Regensburg D-93053, Germany. ulrike.strauch@klinik.uni-regensburg.de
Telephone: +49-941-9447001 Fax: +49-941-9447002
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Abstract

AIM: To investigate the effects of ZK1916784, a low calcemic analog of calcitriol on intestinal inflammation.

METHODS: Acute and chronic colitis was induced by dextran sodium sulfate (DSS) according to standard procedures. Mice were treated intraperitoneally with ZK1916784 or placebo and colonic inflammation was evaluated. Cytokine production by mesenterial lymph node (MLN) cells was measured by ELISA. Immunohistochemistry was performed to detect intestinal dendritic cells (DCs) within the colonic tissue, and the effect of the calcitriol analog on DCs was investigated.

RESULTS: Treatment with ZK191784 resulted in significant amelioration of disease with a reduced histological score in acute and chronic intestinal inflammation. In animals with acute DSS colitis, down-regulation of colonic inflammation was associated with a dramatic reduction in the secretion of the proinflammatory cytokine interferon (IFN)-γ and a significant increase in intereleukin (IL)-10 by MLN cells. Similarly, in chronic colitis, IL-10 expression in colonic tissue increased 1.4-fold when mice were treated with ZK191784, whereas expression of the Th1-specific transcription factor T-beta decreased by 81.6%. Lower numbers of infiltrating activated $CD11c^+$ DCs were found in the colon in ZK191784-treated mice with acute DSS colitis, and secretion of proinflammatory cytokines by primary mucosal DCs was inhibited in the presence of the calcitriol analog.

CONCLUSION: The calcitriol analog ZK191784 demonstrated significant anti-inflammatory properties in experimental colitis that were at least partially mediated by the immunosuppressive effects of the derivate on mucosal DCs.

Key words: Dextran sodium sulfate colitis; Calcitriol; Colonic inflammation; Dendritic cells

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic immune-mediated disease of the gastrointestinal tract of still unknown etiology, most commonly involving inflammation of the terminal ileum and colon. The disease is characterized by deregulated immune responses, and in particular, $CD4^+$ T cells that produce large amounts of proinflammatory cytokines [such as interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF-α)] have been shown to play a central role[1]. Besides genetic factors that are thought to predispose individuals to develop IBD[2], the environment seems to contribute to the disease[3,4]. There is reason to believe that vitamin D may be an environmental factor that affects IBD, as vitamin D deficiency has been linked to ulcerative colitis and Crohn's disease even when the disease is in remission[5,6]. Additionally, vitamin D deficiency has been shown to accelerate the development of IBD symptoms among IL-10 knockout mice that develop spontaneous enterocolitis[7]. Also, calcitriol treatment reduces the ability of IL-10 knockout mice to produce and to respond to

TNF-α[8], and it has recently been shown that application of a calcitriol analog reduces intestinal inflammation in a 2,4,6-trinitrobenzene sulfonic acid colitis model[9].

Calcitriol is the activated form of vitamin D3 that binds to a nuclear receptor protein. In the $CD4^+CD45RB^{high}$ transfer model of colitis, in which T- and B-cell deficient recipient mice develop IBD symptoms when injected with $CD4^+CD45RB^{high}$ T cells, lymphocytes from vitamin D receptor (VDR) knockout mice increase colitis severity in recipient mice compared to similar cells from wild-type animals[10]. Also, VDR knockout mice develop a more severe colitis in response to dextran sodium sulfate (DSS) treatment than wild-type mice, which can be decreased in severity by dietary or rectally administered calcitriol[11]. To underline the importance of calcitriol in IBD, the VDR gene has been mapped to a region on chromosome 12 that has been shown to be linked to susceptibility for Crohn's disease, which suggests an influence of calcitriol on the intestinal immune system[12].

The immunoregulatory effects of the cellular receptor for calcitriol are complex and not yet finally understood. However, as the VDR was discovered in resting and activated lymphocytes[13], it has been suggested that calcitriol might be involved in immunoregulation. In particular, $CD4^+$ T cells that are known to play a major role in chronic colitis express VDR, and are among the identified targets of activated vitamin D[14,15]. Indeed, calcitriol is able to inhibit T-cell proliferation and secretion of different proinflammatory cytokines *in vitro*[13,16], and to suppress the development of several experimental models of chronic inflammatory diseases, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis and diabetes *in vivo*[7,10,17-19]. Also, the presence of calcitriol significantly inhibits cell proliferation of T lymphocytes obtained from patients with ulcerative colitis[20,21]. In mice treated with calcitriol, increased production of TGF-β1 and IL-4 was observed[22], cytokines that are associated with the inhibition of T cell effector function in a murine model of colitis[23]. Furthermore, calcitriol has been found to inhibit dendritic-cell (DC) maturation, which leads to reduced alloreactive capacity[24] and enhanced generation of regulatory $CD4^+CD25^+$ T cells[25].

Calcitriol selectively regulates the immune response without compromising host ability to fight infection[17], which makes it an attractive target compared to standard immunosuppressive medication in patients with IBD. However, calcitriol has clear dose-limiting hypercalcemic effects that interfere with its systemic clinical use due to strong influence on calcium homeostasis and the risk of associated side effects. Recently, ZK191784, a new low-calcemic calcitriol derivative, has been identified that inhibits lymphocyte proliferation and suppresses secretion of proinflammatory cytokines by monocytes[26]. The compound has shown immunosuppressive activity in a murine contact hypersensitivity model when given systemically at concentrations that do not cause hypercalcemic effects, thereby suggesting a possible therapeutic application as an immunosuppressive agent[26].

The aim of our study was to investigate whether this modified calcitriol analog might have potential therapeutic value in the treatment of acute and chronic intestinal inflammation, using the DSS model of colitis.

MATERIALS AND METHODS

Mice

Female Balb/c mice were obtained from Charles River (Germany) and were used for the experiments at 6-8 wk of age and 20-22 g body weight. Animals obtained food and water *ad libitum*. The local Institutional Review Board approved the animal studies.

Reagents and antibodies

The calcitriol analog ZK191784 was synthesized as previously described[26], dissolved in ethanol at a concentration of 1×10^{-2} mol/L and kept at -20℃ until use. The following antibodies were used in the study (all purchased from BD Pharmingen, Heidelberg, Germany): anti-CD3, anti-CD11c, anti-CD28, anti-CD16/CD32, anti-MHC-Ⅱ, anti-CD40, anti-CD80, and anti-CD86.

Induction and treatment of DSS colitis

DSS (molecular mass 40 000) was purchased from ICN (Eschwege, Germany). Acute colitis was induced by feeding 3% DSS over 7 d[27]. Treatment with ZK191784 (100 μg/kg per day orally) or vehicle was either started on day -3 before DSS administration and maintained through to d 7 ("pretreatment"), or started on the first day of DSS application through d 7 ("treatment"). Mice were sacrificed on d 8. For induction of chronic colitis, mice received four cycles of DSS treatment. Each cycle was followed by a period of 10 d water without DSS. Treatment was performed before the first and before the third cycle of DSS for 7 d each (100 μg/kg per day orally). The animals were killed on d 8 after completion of the fourth cycle.

Histological examination

From the distal third of the colon, 1 cm of tissue was harvested from each animal, embedded in paraffin, stained with hematoxylin/eosin after sectioning, and used for histological analysis. To quantify the tissue damage, a scoring system was used as described previously[27]. Thereby, three sections, each obtained at a distance of 100 μm were evaluated. Mice were scored individually, each score representing the mean of three sections. Two independent investigators, blinded to the treatment group, performed histological examination. The total histological score represented the sum of the epithelium and infiltration score, and ranged from 0 to 8[27].

Cytokine ELISA of mesenterial lymph node (MLN) cells

MLN cells (pooled from each group of mice) were collected in cold cell-culture medium [RPMI-1640, 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin; GIBCO-BRL, Eggenstein, Germany] and β-mercaptoethanol (3×10^{-5} mol/L; Sigma). Tissues were mechanically disrupted and the cell suspensions were filtered through a cell strainer (70 μm). Tissue-culture plates were coated in part with anti-CD3 (2.5 μg/well), and 2×10^5 cells/well were incubated in 200 μL

complete medium containing 10 U/mL IL-2 (Proleukin; Chiron) and in part with soluble anti-CD28 (1 μg/mL) for 24 h. Cytokine levels were measured in the supernatant by ELISA (all from Endogene, Woburne, MA, USA), according to the manufacturer's instructions.

Quantitative RT-PCR (light cycler)

RNA was extracted from colonic tissue using the RNeasy kit (Qiagen, Hilden, Germany) and transcribed (Promega, Mannheim, Germany). Quantification of cytokine mRNA was performed using a light cycler (Roche, Molecular Systems, Mannheim, Germany). The following primer pair was used for amplification of T-beta: 5'-AGGCTGCCTGCAGTGCTTC-3'and 5'-CTCGC CTGGTGAAATGTGC-3', annealing temperature at 62℃, and 3 mmol/L $MgCl_2$. For IL-10 amplification, the following primer pair was used: 5'-TCCTTAATGCAG GACTTTAAGGGTTACTTG-3'and 5'-GACACCTTG GTCTTGGAGCTTATTAAAATC-3', annealing temperature 62℃, and 3 mmol/L $MgCl_2$. For standardization, β-actin was amplified using the following primer pair: 5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'.

Generation of bone-marrow-derived DCs and isolation of $CD11c^+$ primary DCs

Bone-marrow-derived DCs (BM-DCs) were generated as described previously[28]. Briefly, bone marrow was flushed from femurs and tibiae of mice, and cells cultured for 10 d with PRMI (RPMI, 10% FCS, 1% penicillin/streptomycin, 1% l-glutamine, and 0.1% 2-mercaptoethanol) containing 200 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech/Tebu, Germany). Subsequently, BM-DCs were incubated with different concentrations of ZK191784 (10, 100 and 1000 nmol/L) and stimulated overnight with 1 μg/mL LPS (Sigma, Germany). Supernatants were harvested for ELISA and BM-DCs were used for fluorescence-activated cell sorting (FACS) analysis.

For isolation of $CD11c^+$ DCs, spleen and MLNs were excised from ZK191784- or vehicle-treated animals (100 μg/kg per day orally, for 5 d), injected with 100 U/mL collagenase Ⅳ in RPMI (Worthington Biochemicals, Lakewood, NJ, USA) and incubated for 30 min at 37℃. After incubation, organs were mechanically dissociated. In the case of the spleen, red blood cells were removed by ACK lysis. $CD11c^+$ DCs were positively selected from single-cell suspensions from the spleen and MLNs using CD11c microbeads and a magnetic cell separation column, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting DC preparations were > 95% CD11c-positive. Isolated DCs were stimulated overnight with 5 μg/mL phosphothioate-stabilized CpG-ODN with the following sequence: ODN1668 5'-TCCATGACGTTCCTGATG CT-3' (Metabion, Martinsried, Germany). Supernatants were harvested for ELISA and cells were used for FACS analysis.

FACS analysis and immunohistochemistry

Cells were analyzed by FACS using two-color staining. Briefly, isolated lymphocytes were incubated with 20 μg/mL of anti-CD16/CD32 mAb and 10% FCS to block Fc receptors, and stained with both fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated mAbs. The cells were washed and analyzed by flow cytometry using an EPICS-XL MCL Coulter. For immunohistochemistry, intestinal tissue samples were snap-frozen in liquid nitrogen embedded in optimal cutting temperature medium and 5- to 10-μm cryostat sections were cut. Incubation with primary antibodies was followed by incubation with biotinylated polyclonal anti-rat IgG or anti-hamster IgG (both Dianova, Germany) mAbs as secondary antibodies. Tissue was stained using the avidin/biotin complex immunoperoxidase kit according to the manufacturer's instructions (Vector Laboratories) and developed with 3-amino-9-ethylcarbazol. Sections were counterstained with hematoxylin/eosin.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U* test for unpaired samples (histological score, cytokine levels) and Student's *t* test (cytokine levels of primary DCs and BM-DCs). Differences were considered statistically significant at $P < 0.05$ (labeled). For graphical analysis of the data, box plots were used. The box stretches from the lower hinge to the upper hinge and contains the middle half of the scores in the distribution. The median is shown as a line across the box, and the largest value below the upper hinge and the smallest value above the lower inner fence are drawn additionally, indicating the distribution.

RESULTS

Treatment with ZK191784 ameliorates acute and chronic DSS-induced colitis

As shown previously, vitamin D deficiency leads to severe colitis in IL-10 knockout mice[7]. However, limitations are involved in the study of cytokine-deficient animals, as intestinal inflammation in these mice may not represent a normal immune response, due to the lack of important regulatory cytokines. Therefore, we decided to investigate the effects of ZK191784 in the DSS models of acute and chronic colitis, as results in both models are not impaired by cytokine deficiency.

Administration of ZK191784 to mice before and during induction of acute colitis ameliorated colitis, as demonstrated by a significant reduction in the histological score by 25% compared to control mice (Figure 1A: control, 7.2 ± 0.44 *vs* ZK191784, 5.4 ± 1 34; $P < 0.05$), and reflected by a less severe inflammatory infiltrate and reduced epithelial damage, as seen in histological sections of the intestine (Figure 1C). Whether animals were pretreated with the calcitriol analog before induction of colitis or whether application of ZK191784 was started at the time of colitis induction made no difference in the ameliorating effect of the compound. Also, when animals were treated sequentially with the calcitriol analog during induction of chronic colitis, intestinal inflammation improved as well by 27.1% (Figure 1B: control, 7. 2 ± 1. 2 *vs* ZK191784, 5.25 ± 1. 9; $P < 0.05$). As demonstrated previously[26], the calcitriol compound did not increase serum levels of calcium in a significant manner, as compared to serum calcium levels in control animals (data not shown).

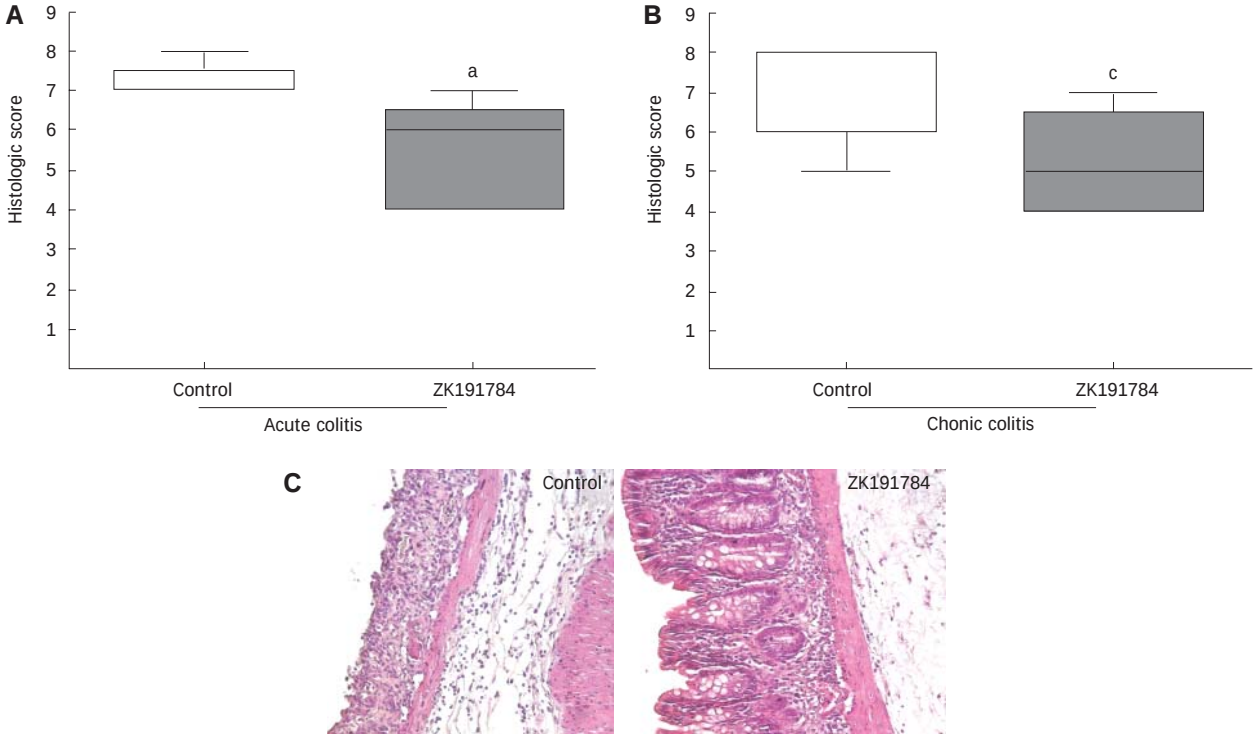

Figure 1 Treatment with ZK191784 ameliorates intestinal inflammation in acute and chronic DSS colitis. **A**: Acute DSS colitis was induced by administration of 3% DSS for 7 d. [a]$P < 0.05$, $n = 5$ mice per group. Data presented are representative of three independent experiments; **B**: Administration of ZK191785 was started after the first and the third cycle of DSS administration for induction of chronic colitis. [c]$P < 0.05$, $n = 8$ or 9 mice per group. Data show one of two independent experiments. Data are displayed as box plots in which the box contains the middle half of the scores in the distribution, the median is shown as a line across the box, and the largest value below the upper hinge and the smallest value above the lower inner fence are indicate the distribution; **C**: Representative colonic hematoxylin/eosin sections from animals with acute DSS colitis treated with ZK191784 or vehicle (× 100).

Treatment with ZK191784 suppresses the secretion of proinflammatory cytokines by MLN cells

In order to further characterize the differences in colitis severity between ZK191784-treated and control animals, we compared the cytokine secretion in both groups. Therefore, MLNs of diseased animals were harvested at the end of the experiment, and levels of pro- and anti-inflammatory cytokines within the supernatant of MLN cells were measured. The secretion of the proinflammatory cytokines IFN-γ (Figure 2A) and IL-6 (data not shown) by isolated cells was dramatically suppressed by treatment with ZK191784 in acute colitis compared with control mice (IFN-γ, -62.7%; IL-6, -47.4%, $P < 0.05$), whereas IL-10 secretion increased significantly by 2.3-fold (Figure 2B). Similarly, in chronic colitis, spontaneous IFN-γ secretion was suppressed by ZK191784 (control, 46.5 ± 8.4 pg/mL; ZK191784, 4.2 ± 1.2 pg/mL, $P < 0.05$). However, in chronic colitis we were not able to detect a significant difference in the secretion of IL-6 or IL-10 by MLN cells derived from ZK191784- or vehicle-treated animals (data not shown).

Th1 cytokines play a proinflammatory role in chronic DSS-induced colitis[29]. Therefore, we evaluated the effects of ZK191784 administration on expression of the Th1-specific transcription factor T-beta in the colonic tissue, using real-time PCR. As shown, the levels of T-beta expression were significantly reduced to 18% compared with those in the control group ($P < 0.0001$, Figure 2C). Additionally, even if we were not able to detect a significant difference in the secretion of the anti-inflammatory cytokine IL-10, the mRNA-levels of these cytokines increased slightly by 1.4-fold in ZK191784-treated animals ($P = 0.00025$, Figure 2D).

Treatment with ZK191784 prevents infiltration of intestinal lamina propria with activated CD11c$^+$ DC in animals with acute colitis

Vitamin D3 is known to interact with antigen-presenting cells and to prevent their activation[30], therefore, we were interested to see whether administration of the calcitriol compound ZK191784 influenced the activation of intestinal DCs within the inflamed colonic mucosa. As shown in Figure 3, immunohistochemical analysis for CD11c$^+$ DCs within the colonic lamina propria revealed a dramatic decrease in cell numbers in intestinal tissue derived from mice treated with ZK191784, as compared to that from control mice. Additionally, when staining for the activation marker CD80 was performed, almost no positive cells were found in the colonic mucosa of calcitriol-treated animals, whereas as expected, in control animals, large numbers of infiltrating CD80$^+$ cells were detected. No changes were observed concerning the numbers of CD3$^+$ T cells (data not shown).

ZK191784 inhibits the production of proinflammatory cytokines and the expression of costimulatory molecules by in vitro-derived DCs

So far, our results indicated that the positive effects of ZK191784 could be mediated by an influence of the compound on antigen-presenting DCs. It has previously

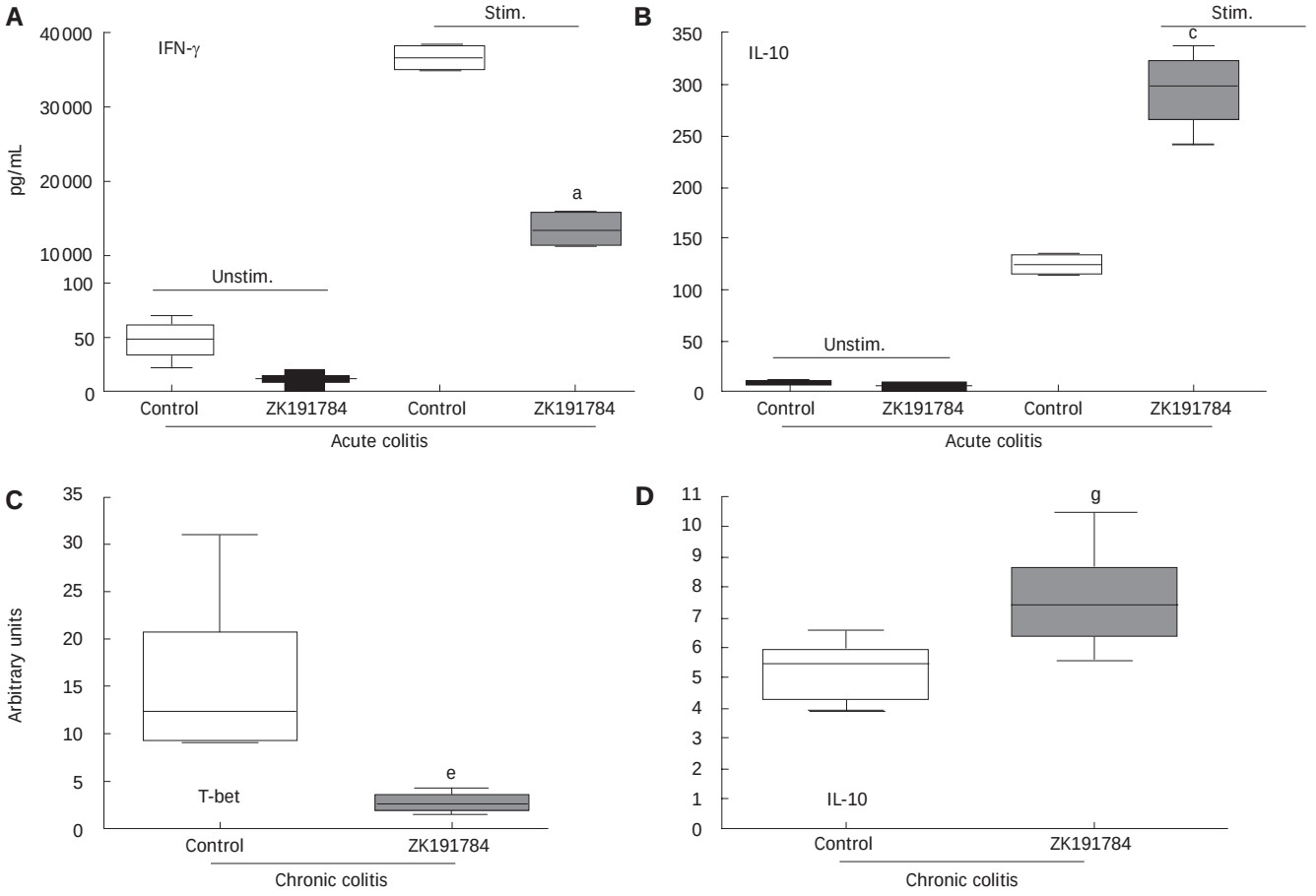

Figure 2 Effects of ZK191784 treatment on cytokine production in acute and chronic colitis. (**A** and **B**) $^{a}P < 0.05$, $^{c}P < 0.05$, $n = 5$ animals. Data presented are representative of three independent experiments. (**C** and **D**) $n = 8$ or 9 animals per group. Data presented show one of two independent experiments. $^{e}P < 0.05$, $^{g}P < 0.05$. Data are displayed as box plots, in which the box contains the middle half of the scores in the distribution, the median is shown as a line across the box, and the largest value below the upper hinge and the smallest value above the lower inner fence indicate the distribution.

been shown that calcitriol inhibits IL-12 production by human monocytes[31]. To determine whether this capacity was shared by ZK191784, the calcitriol analog was tested for its ability to inhibit the secretion of proinflammatory cytokines by BM-DCs that were stimulated with LPS overnight in the presence of different concentrations of ZK191784.

The secretion of IL-10 was not influenced by the presence of the compound (data not shown). However, ZK191784 was able to significantly inhibit the production of the proinflammatory cytokines IFN-γ and IL-12 by 60% and 40%, respectively, in a dose-dependent manner, after stimulation of cells with LPS (Figure 4A and B; IFN-γ, $P = 0.0227$; IL-12, $P = 0.0069$). Additionally, FACS analysis revealed reduced expression of costimulatory molecules (CD40, CD80 and CD86) after stimulation on the surface BM-DCs exposed to ZK191784 (Figure 4C). Therefore, the presence of the calcitriol analog within cell cultures seems to inhibit the activation of *in vitro*-derived BM-DCs.

ZK191784 inhibits the production of proinflammatory cytokines by primary DCs

To further evaluate the effects of ZK191784 on DC *in vivo*, mice were treated with the calcitriol analog or vehicle, and primary CD11c^{+} DCs were isolated from the spleen and MLNs. FACS analysis revealed no difference in

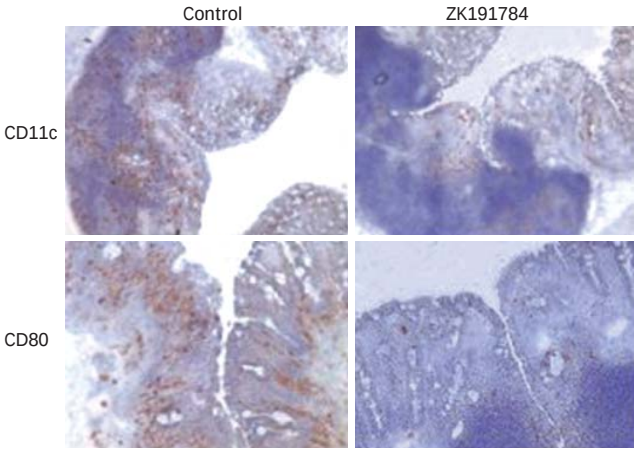

Figure 3 Effects of ZK191784 treatment on the distribution of CD11c^{+} DCs in the colonic mucosa. Sections were stained for CD11c or the costimulatory molecule CD80 (× 100). Staining with isotype controls revealed no background staining (data not shown). Representative sections from one of three mice per group are shown.

the expression of costimulatory molecules in DCs from different treatment groups. However, after stimulation with CpG *in vitro*, primary splenic and mesenteric DCs isolated from mice treated with ZK191784 secreted significantly lower levels of proinflammatory cytokines than those derived from control mice (Figure 5A and B). TNF-α secretion from splenic DCs from ZK191784-

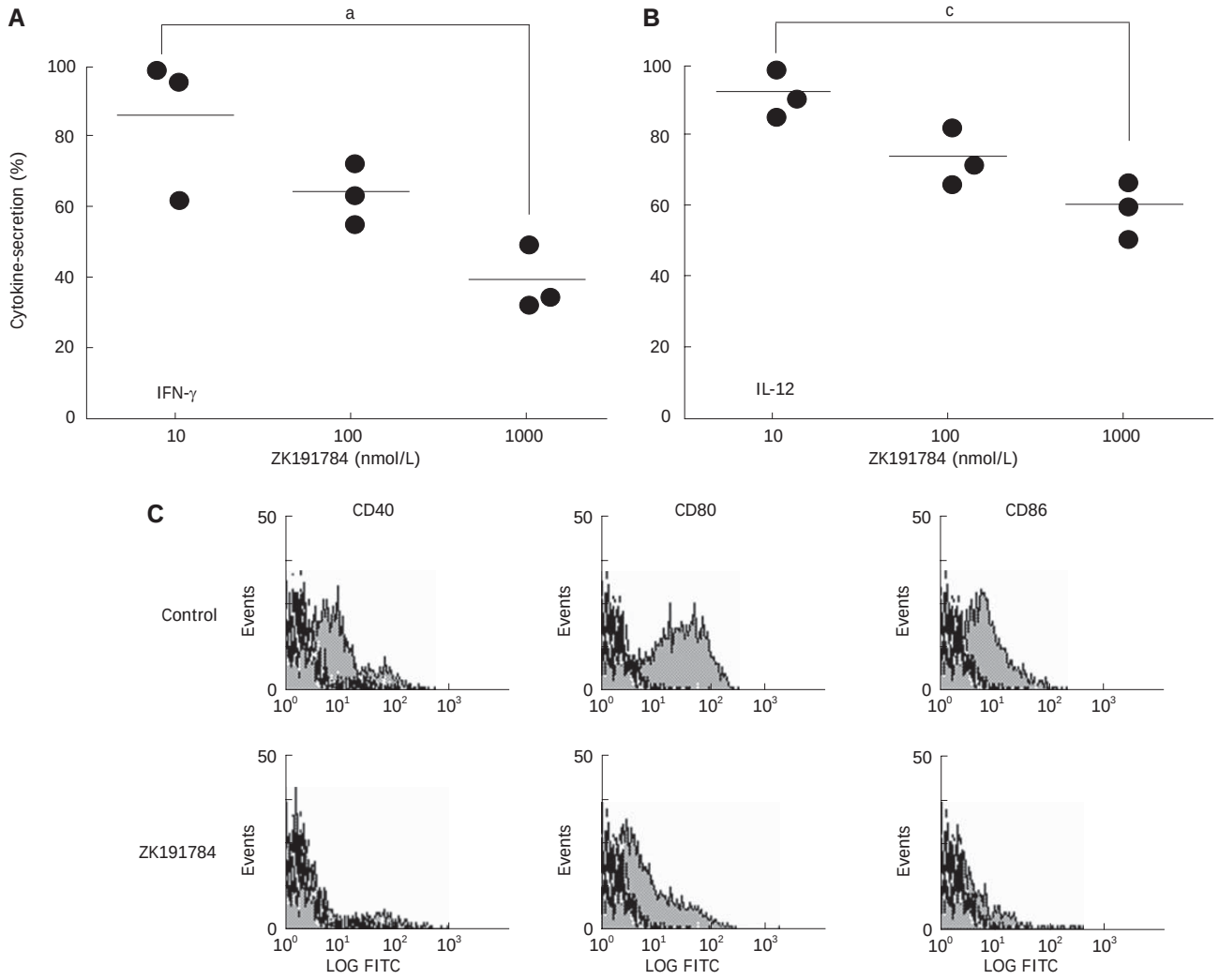

Figure 4 Effects of ZK191784 treatment on the secretion of cytokines and expression of costimulatory molecules by BM-DCs. DCs were generated from bone marrow of mice and stimulated with LPS (1 μg/mL) overnight in the presence of different concentrations of ZK191784 (10, 100 or 1000 nmol/L). Concentrations of the proinflammatory cytokines IFN-γ (**A**) and IL-12 (**B**) were measured within the supernatants by ELISA. $^{a}P < 0.05$, $^{c}P < 0.05$, data presented are representative of three independent experiments. BM-DCs were cultured in the presence of ZK191784 (1000 nmol/L) or vehicle, and were stimulated overnight with LPS. $CD11c^{+}$ DCs were stained with FITC-conjugated mAbs for expression of the costimulatory molecules CD40, CD80 and CD86. Isotype controls are shown with dark lines, and positive staining is shown in grey (**C**).

treated animals was reduced to 74% ($P = 0.0364$) and IL-12 production was decreased to 62%, as compared to control DCs ($P = 0.0136$). The effect was even more dramatic when secretion of proinflammatory cytokines from mucosal MLN DCs was evaluated. We were able to show that TNF-α and IL-12 levels from ZK191784-treated mice were reduced to 9.0% ($P = 0.0005$) and 4.4% ($P < 0.0001$), respectively, compared to cells from control animals. The results suggest that the *in vivo* effects of the calcitriol analog ZK191784 are more dramatic at mucosal sites of the intestine than in systemic lymphatic tissue.

DISCUSSION

In our study we extended previous data about the importance of calcitriol in colitis as we demonstrated that administration of ZK191784, a less calcemic analog of calcitriol, ameliorated not only acute but also chronic DSS-induced colitis. In contrast to previous studies, in which the influence of vitamin D deficiency was investigated in IL-10 knockout mice, our results were not obtained in cytokine-deficient animals, but in normal Balb/c mice. Additionally, our data suggested that the effects of the compound were at least partly due to its influence on DCs in the intestinal mucosa, as systemic administration of ZK191784 inhibited the activation of DCs within the spleen, MLNs and intestinal lamina propria by down-modulation of proinflammatory cytokines and inhibition of costimulatory molecule expression on the surface of DCs.

Calcitriol is known to act directly on T cells because vitamin D3 inhibits the proinflammatory transcription factor nuclear factor (NF)κB, and subsequently impairs the expression of proinflammatory cytokines TNF-α and IFN-γ[20]. TNF-α is known to be an important mediator of inflammation in IBD patients[32], and several TNF-α-blockers are effective in IBD[33]. Therefore, any treatment that targets the TNF-α pathway and Th1-associated cytokines is likely to be a possible treatment for intestinal inflammation. Indeed, stimulated splenic and mesenterial DCs produced lower amounts of TNF-α when mice were treated with the calcitriol analog, and secretion of the

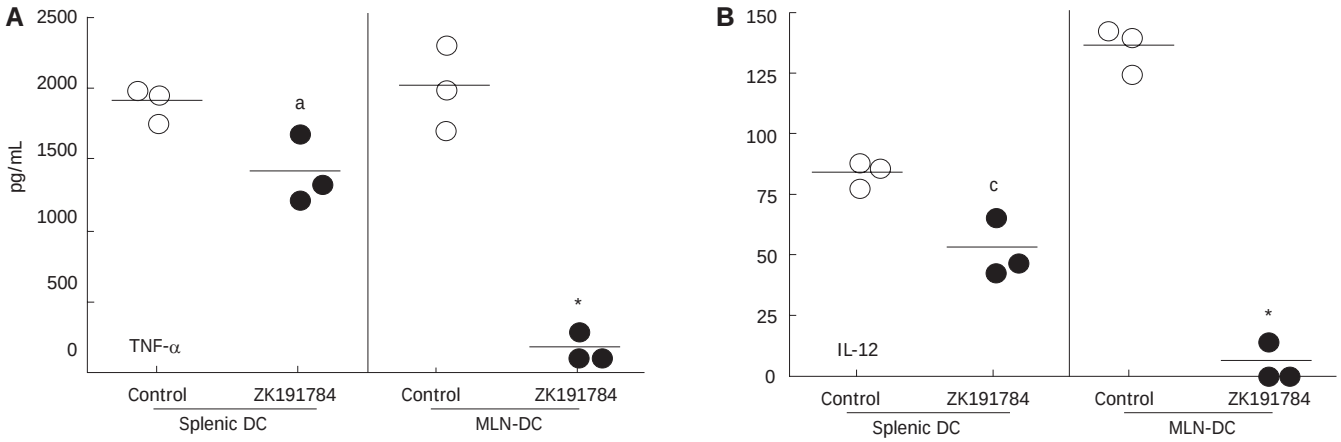

Figure 5 Effects of ZK191784 treatment on the secretion of cytokines by primary DCs derived from spleen and MLNs. $CD11c^+$ DCs were isolated from the spleen and MLNs of animals pretreated with ZK191784 or vehicle. Cells were stimulated overnight with CpG. Triplicate cultures were analyzed for each condition. Concentrations of the proinflammatory cytokines TNF-α (**A**) and IL-12 (**B**) were measured within supernatants by ELISA. $^aP < 0.05$, $^cP < 0.05$, data presented are representative of three independent experiments.

proinflammatory cytokines IFN-γ and IL-6 by MLN cells was dramatically reduced after ZK191784 administration. On the other hand, protein levels of the anti-inflammatory cytokine IL-10 increased in acute colitis and mRNA levels of these cytokines were also enhanced in the chronic colitis model after application of ZK191784.

In vitro experiments have demonstrated that calcitriol renders DCs in a perpetual state of immaturity, as it down-regulates the expression of MHC class Ⅱ and costimulatory molecules[34,35] and decreases the capacity of myeloid human DCs to induce Th1-cell development[36]. Also, it inhibits IL-12 production by both macrophages and DCs *via* inhibition of NFκB activation and transcriptional repression of the IL-12p40 gene[31,36]. The proinflammatory cytokine IL-12 is involved in the pathogenesis of colitis[37], as administration of anti-IL-12 substantially reduces the severity of intestinal inflammation, and mice treated with IL-12 during the application of DSS develop a more severe acute colitis as compared to controls[38]. Therefore, inhibition of IL-12 secretion by antigen-presenting cells can also be an important way to ameliorate disease.

However, so far, it is unclear whether mucosal DCs are also targets for the immunomodulatory activity of calcitriol. Our results demonstrate that infiltrating activated $CD11c^+$ DCs into the intestinal lamina propria were dramatically reduced in number after systemic administration of ZK191784. Additionally, the reduced expression of costimulatory molecules by DCs, accompanied by the inhibition of proinflammatory cytokine secretion, may contribute substantially to decrease DC-dependent T-cell activation within the intestinal mucosa and could largely account for the immunosuppressive properties of this compound on acute and chronic colitis development in our model.

Interestingly, recent data have suggested that *in vivo* macrophages, DCs and epithelial cells are a potential source of calcitriol[39,40]. Therefore, local calcitriol production may keep antigen-presenting cells in an immature state in the healthy intestinal mucosa, and support the suppression of mucosal Th1 cytokine secretion and T-cell proliferation. This mechanism would help to establish tolerance by inhibition of unnecessary inflammatory responses. It is possible that vitamin D deficiency, either due to malnutrition or malabsorption in patients with IBD, is an additional piece of the puzzle that helps to disturb the normal immunoregulation in the gut. Our data support this hypothesis, as administration of the calcitriol analog was able to ameliorate symptoms of DSS colitis by inhibition of activation of mucosal DCs, as demonstrated by lack of expression of costimulatory molecules that would otherwise drive the inflammatory process.

Another recent study suggests an essential function for DCs in programming lymphocyte homing and microenvironmental positioning[41]. The authors were able to demonstrate that calcitriol that was processed by antigen-presenting DCs upregulated the expression of the chemokine receptor CCR10 on the surface of responding T cells, and enabled them to migrate to the skin. On the other hand, the expression of the gut homing adhesion molecule α4β7 and the intestinal chemokine receptor CCR9 on the T-cell surface was suppressed. Therefore, it is possible that, in addition to changes in the phenotype and number of intestinal DCs, ZK191784 can act *via* DCs on T cells within the gut by up-regulation of skin homing chemokine receptors and down-modulation of gut homing molecules. This may reduce the number of infiltrating T cells within the gut during colitis and ameliorate severity of intestinal inflammation. Further studies are under way to investigate the expression of chemokine receptors on T cells in response to the calcitriol analog ZK191784.

The data presented by us and others point to a crucial role of calcitriol-regulated processes in IBD. As no interference by vitamin D compounds with the ability of animals to act defensively against opportunistic infections has so far been shown[17], calcitriol may be an attractive treatment strategy compared to other immunosuppressive reagents used in IBD patients. However, so far, development of hypercalcemia limits calcitriol administration. The new calcitriol analog ZK191784 that was used in our study has previously been shown to have reduced calcemic activity and

therefore, a more favorable therapeutic profile than calcitriol.

In conclusion, we believe that we showed for the first time that ZK191784, a low-calcemic calcitriol analog, significantly ameliorated acute and chronic DSS-induced colitis, most likely due to inhibition of DC activation that prevented development of proinflammatory pathogenic T cells. This less hypercalcemic calcitriol analog is therefore an attractive immunomodulatory agent with few side effects, which, either alone or in combination with other drugs, may have therapeutic applications in the treatment of IBD.

COMMENTS

Background

Chronic IBD in patients still has unknown etiology. However, it is thought that a dysregulated response of antigen-presenting DCs towards bacterial and food antigens within the gut plays a role within the disease process. Currently, treatment of disease consists of immunosuppressive drugs such as steroids and azathioprine, which have significant side effects. We investigated the effects of a calcitriol analog with few side effects for the treatment of murine colitis. Additionally, we were able to show that the calcitriol analog influenced the phenotype and number of DCs within the gut.

Research frontiers

Besides genetic factors that are thought to predispose individuals to develop IBD, the environment seems to contribute to the disease. There are reasons to believe that vitamin D may be an environmental factor that affects colitis, since vitamin D deficiency has been linked to IBD, even when the disease is in remission. Additionally, vitamin D deficiency has been shown to accelerate the development of colitis symptoms among mice that develop spontaneous enterocolitis. It has been shown that calcitriol selectively regulates the immune response without compromising the host's ability to fight infection, which makes it an attractive therapeutic option compared to standard immunosuppressive medication in patients with chronic colitis. However, calcitriol has clear dose-limiting hypercalcemic effects that interfere with its systemic clinical use, due to a strong influence on calcium homeostasis and the risk of associated side effects.

Innovations and breakthroughs

We were able to show that treatment with ZK191784, a low-calcemic calcitriol analog, resulted in significant amelioration of murine colitis in acute and chronic intestinal inflammation. The down-regulation of colonic inflammation was associated with a dramatic reduction in the secretion of proinflammatory cytokines and a significant increase in anti-inflammatory mediators by cells within local lymph nodes. Additionally, lower numbers of infiltrating activated DCs were found in the colon in mice that were treated with the calcitriol analog, and the secretion of proinflammatory cytokines by mucosal DCs was inhibited in the presence of the calcitriol analog.

Applications

We were able to show, as far as we are aware, for the first time that a low-calcemic calcitriol analog significantly ameliorated acute and chronic murine colitis. Inhibition of DC activation prevented development of proinflammatory pathogenic T cells. This calcitriol analog is therefore an attractive immunomodulatory agent with few side effects, which, either alone or in combination with other drugs, may have therapeutic applications in the treatment of IBD.

Terminology

IBD: Inflammatory bowel disease, chronic recurrent colitis in patients with an autoimmune background. DCs: Dendritic cells, cells that capture foreign antigens and present them to lymphocytes, therefore inducing an immune reaction.

Peer review

This paper by Strauch *et al* investigated the properties of ZK191784, a derivative analog of calcitriol (activated form of vitamin D3), with potentially fewer side effects and similar inhibition of T-cell function, to regulate colitis in an experimental mouse model. The results suggest that treatment with this inhibitor decreased the severity of acute and chronic colitis in this model.

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BASIC RESEARCH

Acanthus ilicifolius plant extract prevents DNA alterations in a transplantable Ehrlich ascites carcinoma-bearing murine model

Tridib Chakraborty, Dipak Bhuniya, Mary Chatterjee, Mosiur Rahaman, Dipak Singha, Baidya Nath Chatterjee, Subrata Datta, Ajay Rana, Kartick Samanta, Sunil Srivastawa, Sankar K Maitra, Malay Chatterjee

Tridib Chakraborty, Dipak Bhuniya, Mary Chatterjee, Mosiur Rahaman, Dipak Singha, Kartick Samanta, Sunil Srivastawa, Malay Chatterjee, Chemical Carcinogenesis and Chemoprevention Laboratory, Division of Biochemistry, Department of Pharmaceutical Technology, Jadavpur University, Calcutta 700032, India
Baidya Nath Chatterjee, Sankar K Maitra, Division of Medicinal Chemistry, Indian Institute of Chemical Biology, Jadavpur, Calcutta 700032, India
Subrata Datta, East Calcutta Girls' College, Lake Town, Calcutta 700089, India
Ajay Rana, Cell Signaling Laboratory, Cardiovascular and Cancer Research Institute, College of Medicine, The Texas A&M University System HSC, Temple, TX 76504, United States
Supported by The Council of Scientific and Industrial Research, Government of India, No. 9/96(470)2K5-EMR-I
Correspondence to: Professor Malay Chatterjee, Division of Biochemistry, Department of Pharmaceutical Technology, Jadavpur University, P.O. Box-17028, Calcutta 700032, India. mcbiochem@yahoo.com
Telephone: +91-33-24146393 Fax: +91-33-24146393
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Abstract

AIM: To investigate the chemopreventive efficacy of the Indian medicinal plant *Acanthus ilicifolius* L Acanthaceae in a transplantable Ehrlich ascites carcinoma (EAC)-bearing murine model.

METHODS: Male Swiss albino mice were divided into four groups: Group A was the untreated normal control; Group B was the EAC control mice group that received serial, intraperitoneal (ip) inoculations of rapidly proliferating 2 x 10^5 viable EAC cells in 0.2 mL of sterile phosphate buffered saline; Group C was the plant extract-treated group that received the aqueous leaf extract (ALE) of the plant at a dose of 2.5 mg/kg body weight by single ip injections, once daily for 10, 20 and 30 consecutive days following tumour inoculation (ALE control); and Group D was the EAC + ALE-treatment group. The chemopreventive potential of the ALE was evaluated in a murine model by studying various biological parameters and genotoxic markers, such as tumour cell count, mean survival of the animals, haematological indices, hepatocellular histology, immunohistochemical expression of liver metallothionein (MT) protein, sister-chromatid exchanges (SCEs), and DNA alterations.

RESULTS: Treatment of the EAC-bearing mice with the ALE significantly ($P < 0.001$) reduced viable tumour cell count by 68.34% (228.7 x 10^6 ± 0.53) when compared to EAC control mice (72.4 x 10^6 ± 0.49), and restored body and organ weights almost to the normal values. ALE administration also increased ($P < 0.001$) mean survival of the hosts from 35 ± 3.46 d in EAC control mice to 83 ± 2.69 d in EAC + ALE-treated mice. Haematological indices also showed marked improvement with administration of ALE in EAC-bearing animals. There was a significant increase in RBC count ($P < 0.001$), hemoglobin percent ($P < 0.001$), and haematocrit value ($P < 0.001$) from 4.3 ± 0.12, 6.4 ± 0.93, and 17.63 ± 0.72 respectively in EAC control mice to 7.1 ± 0.13, 12.1 ± 0.77, and 30.23 ± 0.57 respectively in EAC + ALE-treated group, along with concurrent decrement ($P < 0.001$) in WBC count from 18.8 ± 0.54 in EAC control to 8.4 ± 0.71 in EAC + ALE. Furthermore, treatment with ALE substantially improved hepatocellular architecture and no noticeable neoplastic lesions or foci of cellular alteration were observed. Daily administration of the ALE was found to limit liver MT expression, an important marker of cell proliferation with concomitant reduction in MT immunoreactivity (62.25 ± 2.58 *vs* 86.24 ± 5.69, $P < 0.01$). ALE was also potentially effective in reducing ($P < 0.001$) the frequency of SCEs from 14.94 ± 2.14 in EAC control to 5.12 ± 1.16 in EAC + ALE-treated group. Finally, in comparison to the EAC control, ALE was able to suppress *in vivo* DNA damage by abating the generations of 'tailed' DNA by 53.59% (98.65 ± 2.31 *vs* 45.06 ± 1.14, $P < 0.001$), and DNA single-strand breaks (SSBs) by 38.53% (3.14 ± 0.31 *vs* 1.93 ± 0.23, $P < 0.01$) in EAC-bearing murine liver.

CONCLUSION: Our data indicate that, ALE is beneficial in restoring haematological and hepatic histological profiles and in lengthening the survival of the animals against the proliferation of ascites tumour *in vivo*. Finally, the chemopreventive efficacy of the ALE is manifested in limiting MT expression and in preventing DNA alterations in murine liver. The promising results of this study suggest further investigation into the chemopreventive mechanisms of the medicinal plant *A. ilicifolius in vivo* and *in vitro*.

Key words: *Acanthus ilicifolius*; Chemoprevention; DNA strand-breaks; Ehrlich ascites carcinoma; Haematological

indices; Medicinal plants; Metallothionein; Sister-chromatid exchange; Transplantable tumour.

Chakraborty T, Bhuniya D, Chatterjee M, Rahaman M, Singha D, Chatterjee BN, Datta S, Rana A, Samanta K, Srivastawa S, Maitra SK, Chatterjee M. Acanthus ilicifolius plant extract prevents DNA alterations in a transplantable Ehrlich ascites carcinoma-bearing murine model. *World J Gastroenterol* 2007; 13(48): 6538-6548

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INTRODUCTION

Acanthus ilicifolius Linn., popularly known as "Harkach Kanta" belongs to the family Acanthaceae, has typical spinose margins on its evergreen leaves and stipular spines at stem nodes. The common name of the plant is Holy Leaved Acanthus. It is a gregarious, sparingly branched, evergreen shrub, 0.6-1.5 meters in height, common in the tidal swamps of creeks and rivers along the east and west coasts. The leaves are oblong or elliptic, pinnately toothed, acute or truncate and glabrous; its flowers are blue, sessile in opposite pairs and are in terminal crowded or interrupted spikes; the capsules are oblong, 2.5 cm long and are brown; the seeds are broad-ovate, compressed and are 0.6 cm in diameter. It is a plant of marshy habitat distributed widely throughout the mangroves of India including west coasts, Meghalaya and the Andamans and different parts of the Asian countries like Singhal, Burma, China, Thailand *etc*. The plant grows luxuriously by the side of the Ganges in Sunderbans, Hoogly, Howrah and 24 Parganas in West Bengal. The shrub is also planted as a sand-binder along the banks of tidal rivers and lakes. It is a folklore medicinal plant used mainly against rheumatism, paralysis, asthma and snake-bites. A decoction of the plant with sugar candy and cumin is used in dyspepsia with acid eructations. It is also considered to be a diuretic, and is used as a cure for dropsy and bilious swellings. In Goa, the leaves are employed as an emollient fomentation in rheumatism and neuralgia[1]. The leaves are bruised and soaked in water for external application and are also used as an expectorant. The analgesic, anti-inflammatory[2], and leishmanicidal[3] properties of *A ilicifolius* have been documented, whilst Babu *et al* have reported the antioxidant and hepatoprotective properties of the plant[4].

The propensity of cancer cells to show multiple genetic mutations underscores the concept that the carcinogenic process progresses by the accumulation of discrete genetic alterations. Evidence suggests that genomic instability may provide the driving force behind the genetic plasticity characteristic of cancer cells resulting in DNA damage, gene mutation, sister-chromatid exchanges (SCEs)[5], chromosomal aberrations (CAs), and cellular transformation[6,7]. Since SCEs data alone do not necessarily provide either a quantitative or a qualitative estimate of gross structural chromosomal damage, the SCEs assay should be regarded as a complement to rather than a substitute for CAs analysis. Earlier methods for detecting DNA unwinding in alkali have required physical separation of single- from double-stranded DNA using a hydroxyapatite column[8], specific nuclease digestion and precipitation or filter binding[9]. Moreover, radio-labeling of cells was required for detection of the small amounts of DNA involved. In cells where radio-labeling was not feasible, sensitive fluorimetric methods were substituted to permit detection and quantitation of DNA after column or filter separation. We have used a sensitive technique of Fluorimetric Analysis of DNA Unwinding (FADU) according to our established protocol[10] for the estimation of single-strand breaks (SSBs) *in vivo*. Besides FADU, single cell gel electrophoresis (SCGE) or the Comet assay, in particular the alkaline version of the assay, has also become a popular method for sensitive analysis, detection, and quantitation of DNA damage. Damage is detected as DNA strand-breaks, alkali-labile damage, and excision repair sites in individual interphase cells[11].

Our team workers visited the Sunderban area and gathered some information from the local people who used the plant as drugs for the regression of tumour growth with satisfactory results. Since the plant is easily available and information on the effect of this medicinal plant in a tumour-bearing animal model is not yet explored, it has encouraged us to proceed with the following objectives: firstly, to monitor the effect of its aqueous leaf extract (ALE) on mean survival time, liver histology, metallothionein (MT) expression, and haematological status, since the latter plays an important role in immunological and pathophysiological states of the animal; and secondly, to examine the antigenotoxic effects of the ALE in preventing hepatic DNA damage and SCEs and thereby chemoprotection of cells, particularly those undergoing drastic multiplication when transplanted as ascites cells within the peritoneal cavity of the host.

MATERIALS AND METHODS

Animals

Closed colony inbred male Swiss albino mice, 6-7 wk of age and weighing 20-22 g, obtained from the Indian Institute of Chemical Biology (IICB, CSIR, Govt. of India) Kolkata, India, were used throughout the study. The animals were supplied with standard mouse pellet diet (Hindustan-Lever, Mumbai, India) and water *ad libitum*. The recommendations of Jadavpur University "Institutional Animal Ethics Committee" ["Committee for the Purpose of Control and Supervision of Experiment on Animals" (CPCSEA Regn. No. 0367/01/C/CPCSEA) India] for the care and use of laboratory animals were strictly followed throughout the study and the recommendations were in accordance with the National Institute of Health (NIH) guidelines.

Materials

All the chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO) unless otherwise mentioned.

Plant material

Air-dried over-ground parts of the plant *A ilicifolius* were supplied by the United Chemicals and Allied Products

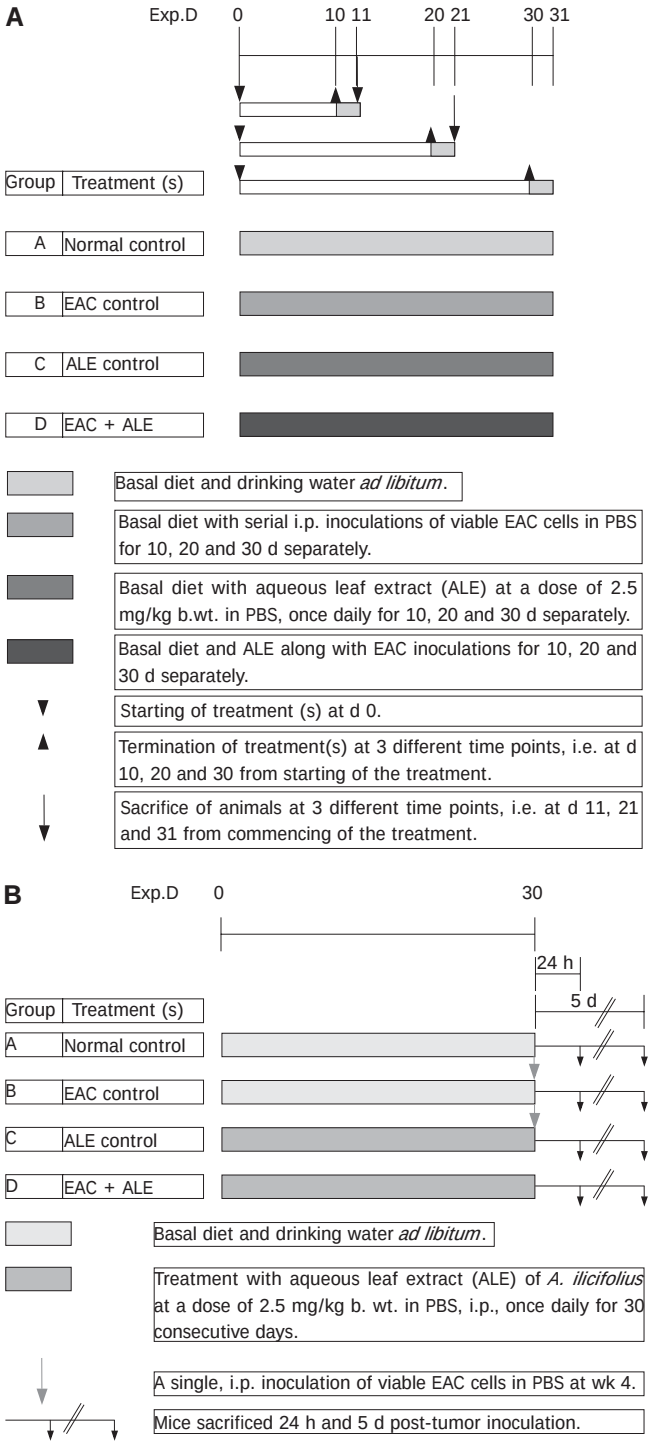

Figure 1 A: Basic experimental protocol for haematology and histology; **B:** Basic experimental protocol for DNA single-strand breaks assay.

(Calcutta, India). Dr. Alpana Bhattacharya, Department of Botany, Bethune College, Calcutta identified and authenticated the plant and a voucher specimen number [B.N.Chatterjee, 100A, 100B (CAL)] was deposited in the Central National Herbarium, Calcutta, India.

Preparation of the aqueous leaf extract (ALE) of the plant

50 g of air-dried powdered leaves of *A ilicifolius* were percolated with distilled water at room temperature until exhausted and filtered, and the filtrate was lyophilized and air-dried (quantity 5.3 g).

Tumour cell inoculation

EAC cells were maintained serially in ascites fluid in the peritoneal cavity of 7-wk-old Swiss albino mice. Viable cells were counted in a haemocytometer by the trypan blue dye exclusion method. An aliquot of 2×10^5 viable cells suspended in 0.2 mL of sterile phosphate buffered saline (PBS, pH 7.4, 0.2 mmol/L) was aseptically inoculated intraperitoneally (ip) into each mouse. The tumour growth was observed within 4-6 d following transplantation.

Experimental Long-term regimen

Mice were randomly divided into four different groups containing 10 mice in each group. Figure 1A shows the basic experimental regimen for haematology and immunohistology. Group A animals belonged to the normal (untreated) vehicle control that received a single, ip injection of 0.2 mL of PBS (pH 7.4) daily for 10, 20 and 30 consecutive days. Group B animals were the EAC-bearing mice (tumour-control group) that received serial ip inoculations of viable tumour cells in PBS. Group C animals were the ALE control group that received daily freshly prepared ALE of *A ilicifolius* in 0.2 mmol/L of PBS by ip administration at a dose of 2.5 mg/kg body weight for 10, 20 and 30 consecutive days of each phase of the study; while group D animals were the treatment group that had been transplanted with viable EAC cells followed by (24 h later) ip administration of the ALE at the same dose-regimen as in group C. The body weight of the animals was monitored after every five days. All the animals were fasted overnight and were sacrificed at three different time intervals separately, i.e. at 11, 21 and 31 d of tumour inoculation to carry out various experiments.

The second set of animals (all the above four groups, A-D) with 20 mice/group was maintained with the same treatment schedule throughout without any prior sacrifice for carrying out survival study.

Short-term regimen

The third set of animals (groups A-D) with 10 mice/group was maintained for SCEs and DNA-chain break studies. In this regimen, the animals were divided into the following treatment schedule (Figure 1B): Group A = Untreated control; Group B = EAC control group that received a single ip EAC inoculation 30 d post-ALE treatment; Group C = ALE control that received the plant extract for 30 d continuously at the same dose-regimen mentioned earlier; Group D = EAC + ALE - treatment group.

Determination of tumour cell count and haematological indices

Tumour cell count in ascites fluid was recorded by the trypan blue dye exclusion method from EAC-bearing mice at a regular interval of 5 d. The total RBC, WBC and haematocrit (Htc) estimations from EDTA - treated blood samples were carried out on d 0, 5, 10 and 15 after tumour transplantation by an improved Neubauer haemocytometer.

Histopathology of liver tissue

Liver slices were taken from each lobe of the liver. After proper fixation and dehydration with graded ethanol

solutions, sections of 5 μm in thickness were stained with haematoxylin and eosin (HE)[12]. The histopathological slides were observed under an ADCON-5591 (ADCON, Cleveland) photomicroscope.

Immunostaining of MT

Immunohistochemical detection of MT in cold acetone-fixed, paraffin-embedded liver sections was performed by the streptavidin-avidin-biotin-peroxidase-complex method[13]. After proper blocking with 1% H_2O_2 and normal goat serum separately, tissue sections were incubated overnight at 4℃ with the primary antibody rabbit anti-rat MT-1 (polyclonal) using a 1:50 dilution. Sections were then incubated with a biotinylated secondary antibody goat anti-rabbit IgG (Sigma) at 37℃ with 1:200 dilution. This was followed by incubation with streptavidin peroxidase (1:100) for 1 h and subsequent chromagen development with 0.5% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and 0.33% H_2O_2 in 0.5 mol/L Tris-NaCl as the substrate. The sections were then counterstained with Harris haematoxylin, dehydrated and mounted and served as positive control. Negative controls were prepared following all the above-mentioned steps omitting the primary antibody. MT immunostaining was considered positive when the nuclei and cytoplasm of the hepatocytes stained prominently purplish brown/reddish brown. MT immunoreactivity was expressed as percentage of immunopositive cells. A total of 10 high power fields were randomly chosen. The number of + ve cells was determined in relation to the total number of cells in that field[13].

SCE analysis

Mice were lightly ether anaesthetized and a vertical incision was made in the lower lateral abdominal region. The subcutaneous tissue was parted with forceps and 5-bromo-2'-deoxyuridine (BrdU) powder (1 mg/kg body wt.) was implanted by the method of Allen *et al*[14]. Approximately 24 h following BrdU implantation, and 1 h after ip injection of 0.04% colchicine at a rate of 1 mL/100 g body wt in 0.9% sodium chloride, mice were sacrificed and liver tissue was processed for chromosome preparation following the procedure of Horiuchi *et al*[15]. Staining for the detection of SCEs was accomplished by a modified fluorescence plus Giemsa (FPG) technique[16].

Scoring of SCEs

In order to determine the SCE levels, approximately 500 well-spread and differentially stained metaphase plates for second division mitosis were scanned from each treatment group, comprising of 10 animals. Each point of exchange was determined as SCE, including clear at the centromere. The SCE analysis was performed at five sequential time points.

Estimation of in vivo DNA damage by the Comet assay

Hepatic DNA damage was measured using the alkaline Comet assay, essentially as described by Olive *et al*[11]. The tissues were homogenized in phosphate-buffered saline (PBS; pH 8.0) under refrigeration and filtered. Cell viability was determined by the Trypan blue method. 4 μL of the homogenized tissue was then transferred to 50 μL of fresh PBS (pH 7.5), washed, suspended in 150 μL of 1% low melting point agarose at 37℃, and pipetted onto an agarose-precoated glass microscope slide. Slides were prepared in triplicate. The slides were immersed for 60 min in freshly-prepared, ice-cold lysis solution (2.5 mol/L NaCl, 0.1 mol/L Na_2EDTA, 10 mmol/L Tris-HCl (pH 10), 10% DMSO and 1% Triton X-100) at 4℃ in the dark, washed, and then subjected to horizontal electrophoresis using freshly made buffer (0.3 mol/L NaOH and 1 mmol/L Na_2EDTA, pH > 13). After electrophoresis, the slides were stained with 5 μg/mL ethidium bromide and viewed under a Zeiss fluorescence microscope equipped with a green excitation filter and a 590 nm barrier filter. Routinely, 150 cells (50 cells/slide) were screened per liver sample from each animal. Nucleoid DNA extends under electrophoresis to form 'comet tails', and the length of the comets was evaluated for determination of the percentage of tailed DNA. This value is linearly related to the frequency of DNA breaks[11].

Assay of DNA unwinding

The principle of fluorimetric analysis of DNA unwinding (FADU) is that the fluorescent dye ethidium bromide (EtBr) binds selectively to double-stranded DNA (DS-DNA) in the presence of single-stranded DNA (SS-DNA) when short duplex regions in SS-DNA molecules are destabilized by alkali treatment[17].

Shearing of DNA, alkali treatment and neutralization

Whole liver genomic DNA was isolated from the frozen murine liver by a modification of the published criteria[18] with enzymatic RNA digestion before proteinase K treatment. After isolation, the purity of DNA solution was checked spectrophotometrically by determining the ratios of absorbance at A_{260}/A_{280} and A_{260}/A_{230}. DNA was sheared by passing the DNA solution (20-25 times) through a 24-gauge needle using a hypodermic syringe. The optical density (OD) of the DNA sample was adjusted to 2.0 at 260 nm. For alkali denaturation, 2.0 mL of DNA solution in Tris-EDTA buffer (20 mmol/L Tris, 1 mmol/L M EDTA, pH 8.0) was mixed with an appropriate aliquot (about 2.4 mL) of alkali solution (0.1 mol/L NaOH, 0.001 mol/L M EDTA), so that the pH of the mixture becomes 12.8. After about 10 min (determined by trial experiments), the pH of the mixture was brought down to about 9.0 by addition of an approximate aliquot (about 1.3 mL) of an acid solution (0.025 mol/L Tris, 0.225 mol/L HCl).

FADU

DNA solutions from control and experimental groups were distributed into 12 test tubes[10]; in each time period (experiments were repeated four times) each tube contained 2.0 mL of the DNA solution of OD equal to 2.0 at 260 nm. The tubes were designated as T, P or B in each group. The DNA solution in tube B was sheared initially as described above. To the P and T tubes, alkali solutions were first added, mixed and then the tubes were incubated at 15℃ for 10 min. Denaturation was stopped by chilling the solutions at 0℃ and addition of acid solution, as described before.

Table 1 Body weight of different groups of mice

Days following tumour transplantation	Body weight (g)			
	Normal control	EAC control	ALE control	EAC + ALE
0	15.6 ± 0.33	15.7 ± 0.46	15.5 ± 0.28	15.6 ± 0.38
10	16.9 ± 0.36	18.4 ± 0.58[a]	17.2 ± 0.41	17.7 ± 0.29
20	18.3 ± 0.26	21.5 ± 0.51[b]	18.5 ± 0.29	19.8 ± 0.35[c]
30	20.6 ± 0.39	25.4 ± 0.66[b]	21.1 ± 0.35	21.7 ± 0.49[d]

Abbreviations: ALE: Aqueous leaf extract of *A ilicifolius*; EAC: Ehrlich ascites carcinoma. Values represent mean ± SE (n = 10). [a]$P < 0.05$, [b]$P < 0.001$ *vs* Normal control; [c]$P < 0.02$, and [d]$P < 0.001$ *vs* EAC control.

Table 2 Final weight of liver and kidney of different groups of animals sacrificed 30 d post-EAC inoculation

Organ (s)	Final weight (g)			
	Normal control	EAC control	ALE control	EAC + ALE
Liver	1.25 ± 0.04	1.13 ± 0.06	1.26 ± 0.08	1.23 ± 0.03
Kidney	0.38 ± 0.03	0.33 ± 0.05	0.38 ± 0.05	0.37 ± 0.04

Abbreviations: ALE: Aqueous leaf extract of *A. ilicifolius*; EAC: Ehrlich ascites carcinoma. Values represent mean ± SE (n = 10). No significant differences between Normal control and EAC control and between EAC control and EAC + ALE.

The T tube differs from P tube in that the alkali and acid solutions, i.e., denaturing and neutralizing solutions were mixed together before addition of DNA solution. An aliquot (0.2 μL) of EtBr solution was added to each tube and the fluorescence was read in a spectrofluorimeter (LS-45, Perkin Elmer, USA) with excitation and emission wavelengths at 525 and 591 nm, respectively. The extent of DNA unwinding after a given time of exposure to alkali is calculated from the fluorescence of T, P and B samples. The fluorescence of the sample less than the fluorescence of the blank (P-B) provide an estimate of the amount of DS-DNA remaining. Percent D is given by the equation: Percent D (DS-DNA %) = (P-B)/(T-B) × 100.

Estimation of SSBs

It is assumed that the distribution of single-strand breaks in the DNA population follows a simple Poisson's Law. Under this circumstance, it is possible to make an approximate estimate of the average number of single-strand breaks (n) per DNA fragment from the simple equation given by Basak[19]: $e^{-n} = D/S + D$; S = percentage DNA that remains single-stranded after alkali treatment; D = percentage remaining as DS-DNA. D/S+D represents the fraction (fo) of the molecules without strand-breaks. The values of 'n' corresponding to different DNA solutions isolated from different groups were then estimated.

Statistical analysis

The data were analyzed using the GraphPad Prism Software package, Version 4.01 (Barcode Softwares, Baltimore, MD). The results were expressed as the mean + SE and Student's t-test was performed to compare sample means. Statistical significance was set at $P < 0.05$ for all comparisons. Percent inhibition was obtained by using the formula [(mean control - mean treatment)/mean control] × 100.

Table 3 Effect of ALE on tumour cell count in Ehrlich ascites carcinoma-bearing mice

Days following tumour transplantation	Mean tumour cell count		Decrease (%)
	EAC control	EAC + ALE	
10	$18.2 \times 10^6 \pm 0.44$	$11.3 \times 10^6 \pm 0.35$[b]	37.91
20	$52.1 \times 10^6 \pm 0.76$	$24.9 \times 10^6 \pm 0.41$[b]	52.20
30	$228.7 \times 10^6 \pm 0.53$	$72.4 \times 10^6 \pm 0.49$[b]	68.34

Abbreviations: ALE: Aqueous leaf extract of *A ilicifolius*; EAC: Ehrlich ascites carcinoma. Values represent mean ± SE (n = 10). [b]$P < 0.001$ *vs* EAC control.

RESULTS

General observations

During the entire period of study, no differences in food and water consumptions were observed among the various groups of animals. Administration of the ALE at a dose of 2.5 mg/kg body weight to the group C animals indicates that the dose was well tolerated with adequate growth responsive effect, otherwise growth retardation or premature death would have occurred. Further, with this particular dose of ALE, there were no toxicologically significant changes in hematology, liver histology, clinical chemistry and clinical enzymology (data not shown), body and relative organ weights *etc*, suggesting that the administered dose of the plant extract was apparently devoid of toxicity.

The body weight of all groups of mice is recorded in Table 1. From the table, it is evident that there was no significant difference in the body weights of the normal control (Group A) and ALE control (Group C) mice suggesting that the plant extract does not have any adverse effect on the growth responses of the host. It can be seen that the body weights of all the four groups (Groups A-D) of animals were more or less the same and did not show any significant differences on the day of tumour transplantation (d 0). On d 10, the body weight of the EAC control (Group B) mice increased significantly ($P < 0.05$) from the normal control (Group A) mice. The body weight continued to increase on successive days (d 20 and 30; $P < 0.01$ and $P < 0.001$ respectively) when compared to the normal control (Group A). In the treatment group (Group D), there was no significant effect of the leaf extract on the reduction of the body weight on d 10 after tumour transplantation. In the following days, there was significant reduction in the body weights ($P < 0.02$ on d 20; $P < 0.001$ on d 30) compared to the EAC control (Group B). The body weights of Group D mice almost came down to normal on d 30 after continuous treatment with the leaf extract.

From Table 2, it is evident that there was no significant difference in liver and kidney weights between the normal control (Group A) and ALE control (Group C) groups suggesting that the aqueous extract probably do not have any adverse effect on the hosts' physiology. Although, there was a slight decrease in the final liver and kidney weights of EAC-bearing mice (Group B) than that of the normal control (Group A) and on the other hand, there was a gradual increase in the organ weights in the treatment group (Group D) than that of Group B,

Table 4 Changes in total count (TC), Hb level and haematocrit in different groups of mice treated with or without ALE

Days following EAC cell inoculation	Group	TC (RBC) (10^6/mm^3)	TC (WBC) (10^6/mm^3)	Hb (g%)	Haematocrit (%)
0	Normal control	7.3 ± 0.12	5.8 ± 0.23	12.6 ± 0.16	33.43 ± 0.16
	EAC control	7.2 ± 0.15	6.5 ± 0.52	12.0 ± 0.31	32.16 ± 0.27
	ALE control	7.3 ± 0.14	5.9 ± 0.22	12.5 ± 0.16	33.58 ± 0.25
	EAC + ALE	7.3 ± 0.13	6.2 ± 0.15	12.2 ± 0.27	33.11 ± 0.23
10	Normal control	7.4 ± 0.14	5.8 ± 0.63	12.6 ± 0.65	33.51 ± 0.32
	EAC control	6.6 ± 0.76	10.4 ± 0.72[b]	10.0 ± 0.78[a]	25.74 ± 0.53[c]
	ALE control	7.6 ± 0.18	5.7 ± 0.55	12.8 ± 0.61	33.75 ± 0.35
	EAC + ALE	6.9 ± 0.53	7.7 ± 0.68[e]	11.0 ± 0.49	29.08 ± 0.69[d]
20	Normal control	7.3 ± 0.54	5.7 ± 0.43	12.4 ± 0.78	33.46 ± 0.17
	EAC control	5.1 ± 0.67[a]	13.9 ± 0.56[b]	8.2 ± 0.92[f]	21.05 ± 0.26[c]
	ALE control	7.5 ± 0.51	5.7 ± 0.38	12.6 ± 0.73	33.71 ± 0.18
	EAC + ALE	6.7 ± 0.12	7.9 ± 0.48[d]	11.5 ± 0.57[h]	28.87 ± 0.35[d]
30	Normal control	7.5 ± 0.14	5.8 ± 0.23	12.7 ± 0.84	33.49 ± 0.16
	EAC control	4.3 ± 0.12[b]	18.8 ± 0.54[b]	6.4 ± 0.93[b]	17.63 ± 0.72[c]
	ALE control	7.7 ± 0.13	5.7 ± 0.24	12.9 ± 0.76	33.65 ± 0.21
	EAC + ALE	7.1 ± 0.13[d]	8.4 ± 0.71[d]	12.1 ± 0.77[d]	30.23 ± 0.57[d]

Abbreviations: ALE: Aqueous leaf extract of *A. ilicifolius*; EAC: Ehrlich ascites carcinoma; TC: Total count. Values represent mean ± SE (n = 10). [a]$P < 0.02$, [b]$P < 0.001$, [c]$P < 0.05$, and [f]$P < 0.01$ *vs* Normal control. [d]$P < 0.001$, [e]$P < 0.02$, and [h]$P < 0.01$ *vs* EAC control.

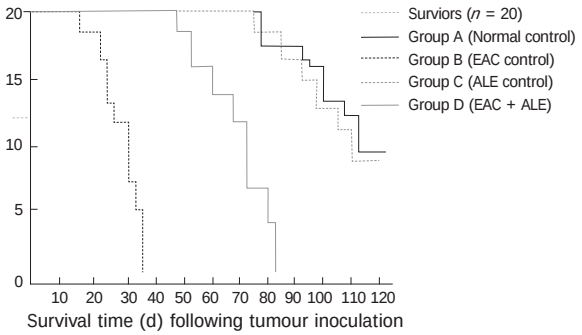

Figure 2 Survival curve of all the four groups of mice.

however all these changes were not statistically significant.

On inoculation of the male Swiss albino mice with EAC (Group B), all mice died of carcinoma within 35 ± 3.46 d of inoculation (Figure 2); while the concurrent administration of ALE to the EAC-bearing hosts (Group D) resulted in an increase ($P < 0.001$) in mean survival of the animals, maximum life-span being 83 ± 2.69 d. Mice from the normal control (Group A) and ALE control (Group C) survived for the entire period of study (i.e. 110 ± 2.12 d and more).

Effect of ALE on mean tumour cell count

The effect of the plant extract on mean tumour cell count is shown in Table 3. No tumour cell was detected on the animals for d 0. Viable tumour cells were observed from d 3/4. There was a gradual increase in cell count in tumour-bearing mice (Group B) from d 10 to d 30. Administration of the ALE to the EAC-bearing mice (Group D) resulted in a gradual significant decrease ($P < 0.001$) in the tumour-cell count from d 10 (37.91%) to d 30 (68.34%).

Effect of ALE on haematological indices

The total count (TC) of RBC, WBC, haemoglobin percent (Hb%) and haematocrit (Htc) of different groups of mice are shown in Table 4. From the table it is evident that, the TC of RBC gradually decreased from d 10 of tumour transplantation in EAC-bearing mice (Group B) and this reduction continued beyond d 30 until the death of the animals. Although the TC of RBC started to decrease from d 10, it showed statistical significance beyond d 10, i.e. on and from d 20 ($P < 0.02$) when compared to the normal vehicle control (Group A). Further reduction was observed on d 30 ($P < 0.001$) in the tumour-bearing animals. In the treatment group (group D), however, administration of ALE restored the TC of RBC significantly ($P < 0.001$ at d 30) almost to the normal value, when compared to Group B (EAC control).

In case of the TC of WBC, the result was just reverse to that of the TC of RBC. The WBC content went on increasing significantly ($P < 0.001$) in Group B since d 10 of tumour inoculation, when compared to the normal control (Group A). Administration of the plant extract significantly reduced the TC of WBC ($P < 0.001$ at d 30) when compared to EAC control (Group B). Furthermore, the Hb% and Htc values were also decreased significantly ($P < 0.001$ at d 30) in EAC-bearing mice compared to the normal control and restored to the normal level in Group D mice after administration of the plant extract.

Effect of ALE on hepatic architecture

Phenotypically altered hepatocyte populations were found scattered in the livers of EAC-bearing mice (i.e. Groups B and D); but no such alterations were noticeable in untreated normal control (Group A; Figures 3A and B) or in the ALE control (Group C, Figure 3F). The HE- stained sections of liver slices revealed extensive hepatocellular lesions that were clearly distinguishable from the non-nodular surrounding normal parenchyma (NNSP).

In Group B (Figures 3C-E), a gross alteration in hepatocellular architecture with neoplastic focal lesions was observed and hepatocytes appeared oval or irregular in shape upon EAC inoculation after 20 d and

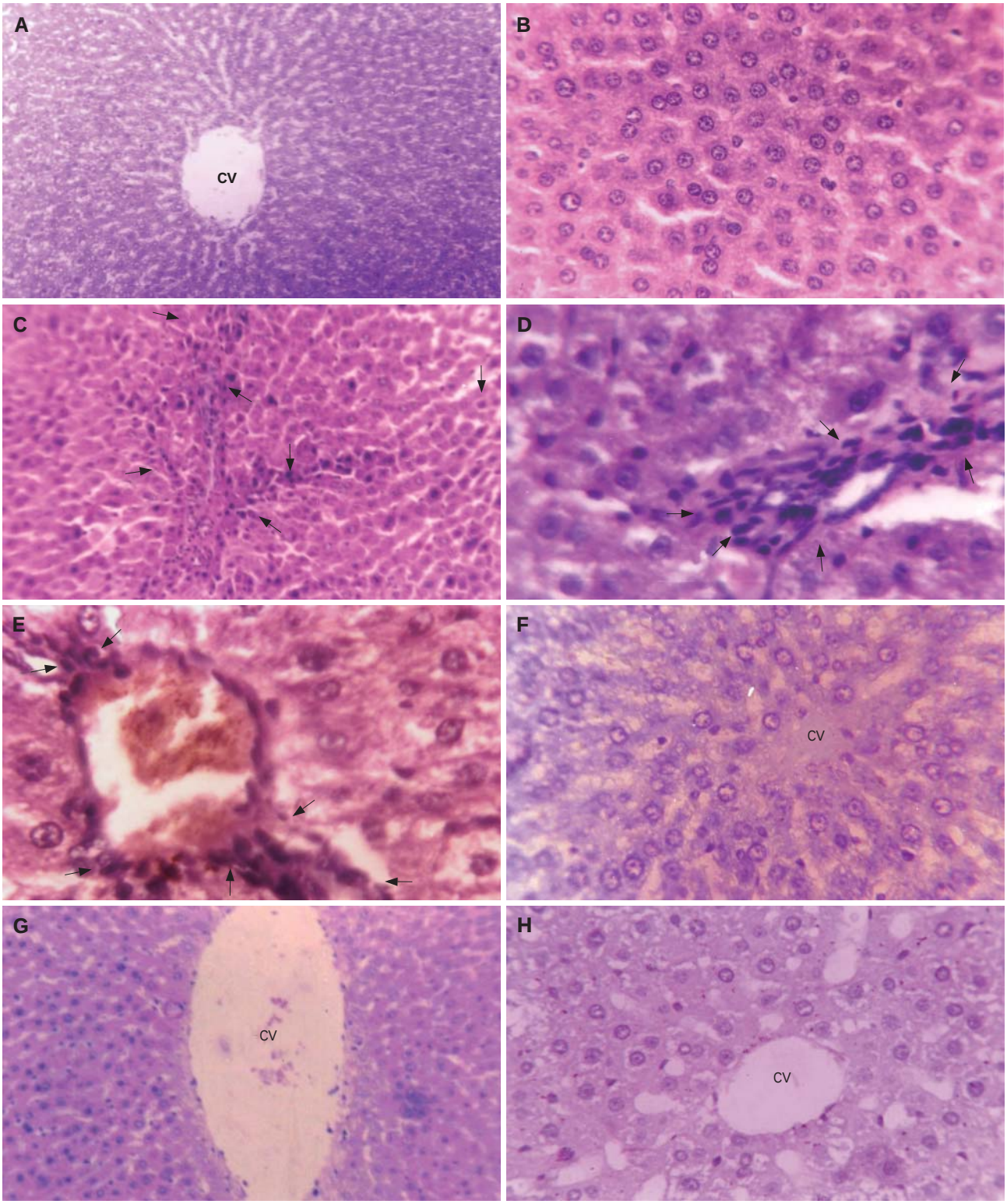

Figure 3 Contiguous liver sections from mice showing hepatocellular histological profiles. **A**, **B**, **F**: Normal hepatocellular architecture depicting hepatocytes radiating from the central vein; **C**, **D**, **E**: Aberrant hepatocellular phenotype with prominent basophilic focal lesions (black arrows) and the presence of eosinophilic and clear cell foci following serial, intraperitoneal (ip) inoculations of viable Ehrlich ascites carcinoma (EAC) cells; **G**, **H**: Almost normal hepatocellular architecture following simultaneous ip administrations of aqueous leaf extract (ALE) of *A. ilicifolius* and EAC. **A** and **B**: Untreated Control; **C**, **D**, and **E**: EAC Control; **F**: ALE Control; **G** and **H**: EAC + ALE. CV: Central vein. Magnification, **A**, **C**, and **G**: × 100; **B**, **D**, **E**, **F**, **H**: × 450.

30 d, with minimal histological changes being noticed after 10 d. The altered hepatocytes were found to be consistently enlarged with more than one nucleus, which were moreover largely vesiculated. They aggregated in clusters resulting in the appearance of prominent basophilic focal lesions. Some nuclei in the cells were large and hyperchromatic with clear and centrally located nucleoli (Figure 3D and E). Extensive vacuolation and necrosis were observed in the cytoplasm with masses of acidophilic (eosinophilic) material after 30 d. In contrast, the cellular architecture of hepatic lobules seemed to be almost like that of normal liver in Group D (Figures

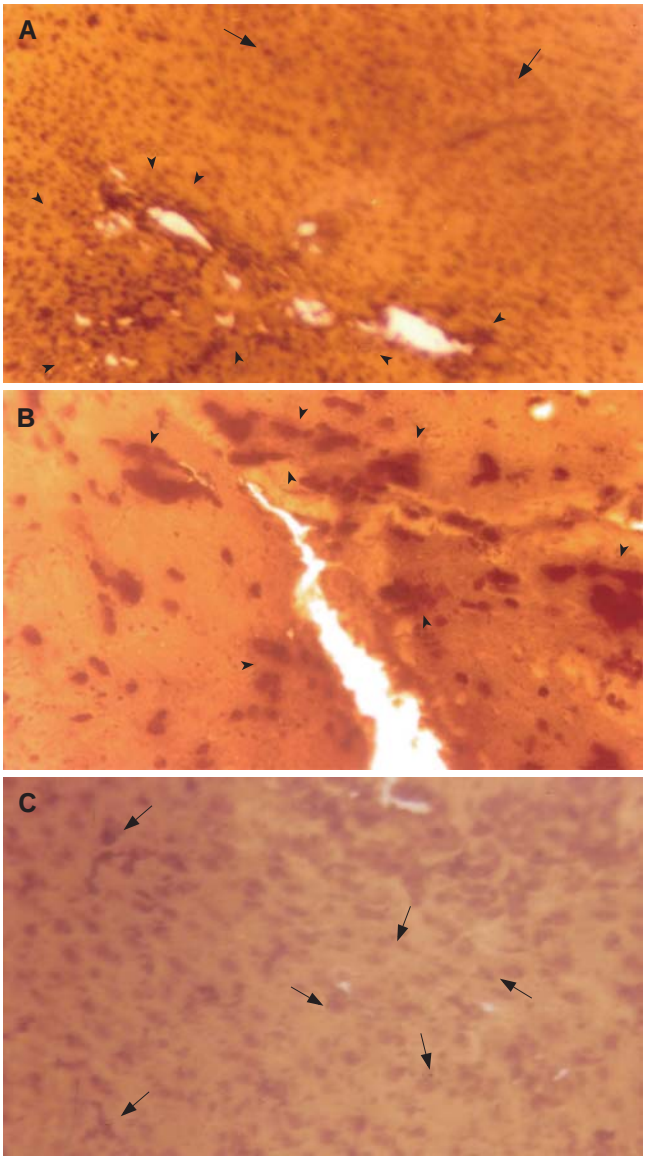

Figure 4 Light micrographs of tissue sections from murine liver (after 30 d of tumour transplantation) showing immunostaining of metallothionein (MT) with anti-rat MT-1 antibody and 3, 3'-diaminobenzidine tetrahydrochloride (DAB). **A** and **B**: Strong immunostaining of MT protein in EAC Control mice; **C**: Reduced immunostaining of MT in EAC + ALE-treatment mice. ALE: Aqueous leaf extract of *A. ilicifolius*; EAC: Ehrlich ascites carcinoma. Arrow Head (▲) indicates intense immunostaining of MT protein with prominent focal expression and isolated clusters of MT-positive cells. Arrow (↑) indicates scattered/individual MT immunopositive cells. Magnification, **A**: × 100; **B** and **C**: × 270.

3G and H) that received ALE treatment during the entire period of study. Liver sections from this group presented only a few altered hepatic cell foci. The cells were generally filled with cytoplasmic material and were less vacuolated. The size of the nuclei was essentially the same as that of normal cells and cells with two nuclei were considerably fewer than in group B.

Effect of ALE on liver MT expression

MT protein was detected *in situ* in the EAC-bearing liver tissue of mice (Group B) depicting a strong immunoexpression (Figures 4A and B). Generally, in sections (Figure 4B) with high MT-immunopositivity, the MT-positive cells formed contiguous foci or sheets and on some occasions isolated clusters of positive cells were

Table 5 Effect of ALE on sister-chromatid exchange (SCE) frequencies (500 plates/group) in different groups of mice killed at various time points

Weeks after tumour inoculation	Group	Mean SCE/cell	Reduction in frequency (%)
1	EAC control	4.59 ± 0.57	-
	EAC + ALE	3.62 ± 0.29	21.13
2	EAC control	6.89 ± 1.13	-
	EAC + ALE	3.78 ± 0.78[a]	45.14
3	EAC control	7.83 ± 1.05	-
	EAC + ALE	4.64 ± 0.54[b]	40.74
4	EAC control	10.26 ± 1.67	-
	EAC + ALE	4.83 ± 0.69[c]	52.92
5	EAC control	14.94 ± 2.14	-
	EAC + ALE	5.12 ± 1.16[d]	65.73

Abbreviations: ALE: Aqueous leaf extract of *A. ilicifolius*; EAC: Ehrlich ascites carcinoma. Values represent mean ± SE (n = 10). [a]$P < 0.05$, [b]$P < 0.02$, [c]$P < 0.01$, and [d]$P < 0.001$ *vs* EAC control.

seen. Figure 4A showed an intense staining of MT protein within the hepatic lesions indicating its focal expression. Semi-quantitative scoring of MT-positive cells showed that, there was substantial elevation of MT-immunoreactivity ($P < 0.0001$) (56.18% and 86.24% respectively at 20 and 30 d post-EAC inoculation; Table not shown) in Group B mice when compared to the basal expression level in normal control mice (Group A). In contrast, sections from ALE-treated murine liver (Group D) showed reduced MT-immunoreactivity [35.86% ($P < 0.05$) and 62.25% ($P < 0.01$) respectively at 20 and 30 d] with scattered MT-positive cells (Figure 4C).

Effect of ALE on the frequencies of SCEs in EAC-bearing mice

With transplantation of EAC, there was a moderate induction ($P < 0.05$) in mean SCE/cell in EAC-control mice (Group B) after 1 wk when compared to the untreated vehicle control (Group A) that showed spontaneous SCEs (2.89 ± 0.39) (Table 5). The induction showed steady increase ($P < 0.02$-0.001) in the frequency of SCEs on and from wk 2 with the progression of tumour development as a function of time and reached the peak (14.94 ± 2.14) prior to the death of the EAC-bearing animals at wk 5. In contrast, ALE administration to the EAC + ALE-treated mice offered a near complete inhibition of SCEs during the early phases of tumour growth. In this group, daily treatment with ALE resulted in a substantial decrement ($P < 0.05$-0.001) in the SCE frequency at all the time intervals (wk 2-5) when compared to EAC control animals at the same time points. Data of wk 2-5 showed 45.14%-65.73% reduction in SCE/cell from EAC + ALE-treated mice. As the mice from EAC control group died after wk 5, therefore, data obtained from the EAC + ALE-treatment group on and from wk 6 were compared with the last available data from EAC control mice at wk 5. Results showed that there were downhill trends in the SCE frequency in the ALE-treated EAC-bearing animals (Group D) until their survival up to wk 12 (data not shown). However, no significant difference between the normal control and ALE control in terms of SCE was noted.

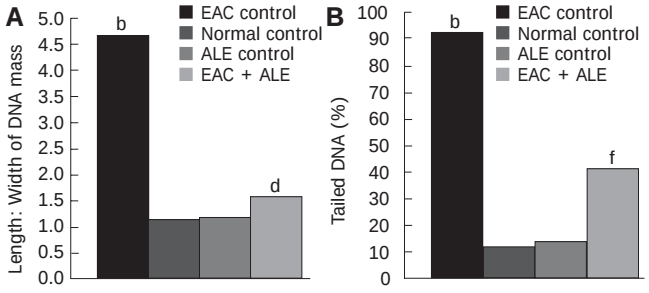

Figure 5 Effect of continuous treatment with ALE on DNA damage in the liver of mice 5 d post-EAC inoculation. **A**: Length:Width of DNA mass; **B**: Percentage of Tailed DNA. Each column and bar indicates the mean ± SE. $n = 10$. $^{b}P < 0.0001$ EAC Control *vs* Normal Control; $^{d}P < 0.01$ EAC + ALE *vs* EAC Control; $^{f}P < 0.001$ EAC + ALE *vs* EAC Control. Abbreviations: ALE: Aqueous leaf extract of *A. ilicifolius*; EAC: Ehrlich ascites carcinoma.

Table 6 Effect of ALE on the generation of average number of single-strand breaks (SSBs)/DNA fragment in murine liver 24 h and 5 d post-EAC transplantation

Time following EAC inoculation	Group	DS-DNA (%)	No. of SSBs/ DNA fragment	Inhibition (%)
24 h	Normal control	94.75	0.07 ± 0.01	----
	EAC control	79.57	1.28 ± 0.22[b]	----
	ALE control	94.87	0.07 ± 0.02	----
	EAC + ALE	88.23	1.14 ± 0.15	----
5 d	Normal control	95.12	0.08 ± 0.03	----
	EAC control	17.21	3.14 ± 0.31[b]	----
	ALE control	94.93	0.07 ± 0.3	----
	EAC + ALE	55.83	1.93 ± 0.23[d]	38.53

ALE: Aqueous leaf extract of *A. ilicifolius;* EAC: Ehrlich ascites carcinoma. Values represent mean ± SE ($n = 10$). $^{b}P < 0.001$ *vs* Normal control; $^{d}P < 0.01$ *vs* EAC control.

Effect of ALE on hepatic DNA damage

The mean length to width (L:W) ratio of the DNA mass, indicating the extent of DNA damage, was increased in the EAC control group 5 d post-EAC inoculation in comparison with the untreated control ($P < 0.0001$; Figure 5A). There was also a significant increase in the frequency of tailed DNA in EAC control mice compared to untreated control ($P < 0.0001$; Figure 5B). Treatment with ALE to the EAC-bearing hosts reduced the L:W ratio of DNA mass (65.34% reduction; $P < 0.01$), and the mean frequency of tailed DNA (53.59% reduction; $P < 0.001$) compared to that of the EAC control group.

Effect of ALE on hepatic SSBs

The protective effect of ALE on SSBs in EAC-bearing hosts 24 h and 5 d post-tumour transplantation is shown in Table 6. In EAC control mice, a significant increase ($P < 0.001$) in the number of SSBs/DNA could be observed following EAC transplantation when compared to that of normal control. The percentage of DS-DNA and SS-DNA in EAC control mice was 79.57% and 20.43%, suggesting that, the extent of host DNA lesions only after 24 h of tumour transplantation was not so severe. Moreover, the amount of DS-DNA (88.23%) in the EAC + ALE-treated group was found to be markedly close to that of normal control (94.75%); whereas, there was almost a 3-fold increase in SS-DNAs (82.79%) and a 4-fold decrease in DS-DNA (17.21%) in EAC control mice sacrificed 5 d post-tumour inoculation, than that of the same group (SS-DNA 31.43%, and DS-DNA 68.57%) being transplanted with tumours only 24 h prior to sacrifice. Treatment with ALE strictly abated (38.53%; $P < 0.01$) the generation of SSBs/DNA fragment in EAC + ALE-treated mice when compared to the EAC control. Moreover, there were no significant differences between normal control and ALE control in the percentage of DS-DNAs, suggesting that ALE apparently is non-genotoxic.

DISCUSSION

The present study showed that administration of ALE reduced viable tumour cell count and brought about a marked increase in mean survival (average life-span) of tumour-bearing hosts, suggesting the tumour-combating efficacy of the said extract under investigation. Furthermore, ALE restored RBC count, Hb and Htc values, and reduced WBC count in EAC-bearing mice, thereby indicating its effectiveness in limiting haematological toxicity following tumour transplantation. There was substantial improvement in the hepatocellular architecture following daily administration of ALE over EAC control mice. We further report here the potential role of the ALE in controlling liver MT expression in mice. Finally, ALE treatment resulted in reduced SCEs and further prevented the generations of 'tailed' DNAs, SS-DNAs and SSBs in EAC-bearing mice hepatocytes as demonstrated by the Comet assay and FADU.

Analysis of the haematological toxicity during tumour transplantation and its possible alteration by ALE was monitored using a battery of haematological indices. Primarily, it resulted in an anemia probably due to severe red cell destruction, which was also reflected in the significantly lowered Htc value as compared to normal animals. The symptoms aggravated with time following tumour transplantation and the body weights of the EAC-bearing mice increased due to the accumulation of haemorrhagous ascitic fluid within the peritoneal cavity. The chemopreventive effects of the plant extract in reducing the severity of anaemia through improvement of RBC count and Htc value[20] not only maintained the normal body and organ weights of tumour-bearing mice, but also may account for the increased survival of the host as evident from our present findings. Administration of ALE further decreased viable tumour cell count in EAC-bearing animals. This may point toward the underlying chemopreventive potential of the plant extract *in vivo*. Although the mechanistic insights into the chemoprevention of the plant are unexplored, we may assume that ALE-mediated tumour cell apoptosis could be one possible pathway for the decreased number of viable tumour cells that warrants further investigation.

The beneficial effect of the plant extract was also apparent in elevating the Hb level, which was otherwise decreased in EAC-control animals. The exact mechanism of the ALE-mediated induction of Hb level during tumour growth is not clear at the present moment. We may speculate that it might somehow influence the process of haem synthesis, which may therefore account for the subsequent induction of Hb level. Whatever may be

the mechanism, induction of Hb by ALE, as observed herein, could have a broad implication with respect to the antitumour efficacy of the extract if one considers the fact that high Hb level has been found to possess an inhibitory influence on tumour growth[20,21]. The data described herein showed a significant increase in total leukocyte count at different time intervals following tumour inoculation as compared to normal animals. Abnormal mitotic activity as evident from the appearance of different forms of abnormal and aberrant neutrophils in large numbers was essentially observed in EAC-bearing hosts. Now, the role of ALE in reducing the TC of WBC in the treatment group could be influenced through a reversal of lymphoid-myeloid ratio. Thus, the stabilization of leukocyte count with parallel retainments of normal Hb level and Htc value in tumour-bearing animals by ALE may have a paramount importance in limiting hematological toxicity in hosts.

The study further demonstrates an intense immunostaining of MT in EAC-bearing liver tissue of mice in comparison to that of normal control. It is necessary to mention that the hyperbasophilic cells of the hepatocellular lesions stained more intensely for MT compared to that of the non-basophilic area of the same tissue, suggesting that the hyperbasophilic proliferative lesions consisting of rapidly proliferating cells may be considered to be the primary MT (-positive focal) expression zones. Up-regulation of MT expression in rapidly proliferating tissues appears to suggest its critical role in normal and neoplastic cell growth[13,22]. Treatment with ALE substantially reduced the expression of MT in the precancerous lesions along with decreased MT immunoreactivity. The precise mechanism of ALE-mediated down-regulation of MT expression is not clear at the moment. However, it is assumed that the antioxidant property[4] of *A. ilicifolius* may be involved in the elimination of reactive metabolites from the host cells, thereby posing low oxidative stress to the hosts and limiting MT expression thereby. Thus, control of liver MT expression, a cell-cycle dependent protein in EAC-transplanted mice by treatment with ALE might play an important role in controlling cell growth *in vivo*.

Inoculation of the hosts with EAC may act as primary agents producing secondary DNA cleavage and are potent inducers of chromosomal aberrations and SCEs[23]. Since a SCE represents the breakage of four strands of DNA (two double helices), a switch of these strands between chromatids of the same chromosome and the rejoining of these strands in their new location, it is important to know whether the breakage and rejoining events occur faithfully, that is without producing any modification in the genetic code. In early experiments using Chinese hamster cells in culture, it was found that the induction of SCEs was linearly related to the increase in single gene mutations when the cells were exposed to chemicals, each of which differed in the type of lesion produced in DNA[23,24]. Reports indicate that antioxidants suppress clastogenicity, and thus, many antioxidants are even anticarcinogens[25,26]. Studies have shown that *A ilicifolius* possesses antioxidant properties[4], and may therefore be effective in combating oxidative stress following tumour transplantation. In our present study, ALE-mediated suppression of SCEs indicates its anticlastogenicity and chemoprotection thereby. The decreased occurrence of SCEs may indirectly be related to increased cell survival or host's anti-cancer surveillance, since studies in search of a relationship between SCE induction and other expressions of genotoxicity have shown a positive relationship between SCEs and reduced cell survival.

DNA damage in the form of SSBs may play an essential role in the pathogenesis of neoplasia. The SSBs detected herein by the Comet assay and FADU can be converted to double-strand DNA breaks (DSBs) upon replication. DSBs are a highly deleterious form of DNA damage, since they lead to mitotic cell death or mutations[27]. DNA DSBs are generated when the two complementary strands of the DNA double helix are broken simultaneously at sites that are sufficiently close to one another that base-pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed. As a consequence, the two DNA ends generated by a DSB may become physically dissociated from one another, resulting in error-prone repair and providing the opportunity for inappropriate recombination with other sites in the genome. Error-prone/inaccurate repair or lack of repair of DSBs may lead to mutations or to larger-scale chromosomal aberrations and genomic instability[28,29]. In addition, mutations in many of the factors involved in DSB signaling and repair lead to increased predisposition to cancer in humans and in animal models[30]. In the present study, ALE treatment resulted in a substantial decrease in the amount of SS-DNAs, SSBs, 'tailed' DNA and DNA 'comets' in the tumour-bearing murine livers. This reduction in DNA damage reflects the potential of the ALE to reduce the genotoxicity caused by EAC in host cells. The exact mechanism of action of the ALE in limiting DNA damage warrants a more robust investigation.

In conclusion, the results of our study clearly indicate that the ALE of *A ilicifolius* is able to inhibit the survival and proliferation of EAC cells in laboratory mice, and this observation is potentially useful in medical oncology. Our data show that, ALE treatment is beneficial in increasing the mean survival of the animals, in remodeling of the liver lesions and in limiting clastogenicity and genotoxicity *in vivo*. Although this study is at its preliminary stage, the results are promising. Future studies are planned in our laboratory to isolate and characterize the active fractions/principles of the plant and to elucidate the mechanistic basis of chemoprevention in a defined chemical carcinogenesis model.

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COMMENTS

Background

Herbal preparations/natural plant products constitute an important component of indigenous/traditional medicines. Herbs certainly have been an important source of many allopathic medicines. However, the way herbs are used in indigenous medical system is not supported by strong preclinical data with insights into the biological/pharmacological mechanisms. Ehrlich ascites carcinoma (EAC) is a strain-specific, rapidly proliferating transplantable epithelial tumour in mouse in which the neoplastic cells multiply and ascites fluid accumulates within the

peritoneal cavity of the host and ultimately the host dies. Inhibition of the growth and proliferation of EAC cells by administration of plant products would be a safe and effective treatment strategy.

Research frontiers

The most significant approach to cancer chemoprevention seems to be the administration of chemopreventive agents in order to inhibit the pathogenesis of cancer or to delay or halt or limit the progression of neoplastic transformation. With the increasing trend in the incidence of cancers in our country, biomedical research directed at early detection and diagnosis, prognosis and survival as well as prevention of progression of malignancy is of prime importance. Since India is a rich source of indigenous herbs and several studies have reported potential therapeutic/chemopreventive utilities of these medicinal herbs, it is therefore of great importance for us to explore the chemopreventive efficacy of an Indian medicinal plant *Acanthus ilicifolius* in preventing DNA damage in an EAC model in mice.

Innovations and breakthroughs

In this paper, the primary chemopreventive mechanisms of the plant *Acanthus ilicifolius* have been investigated in an *in vivo* EAC model in Swiss albino mice. In contrast to meager previous reports demonstrating analgesic and antioxidant properties of the plant, the results of the present study clearly showed that, the aqueous leaf extract (ALE) was quite effective in preventing hepatic DNA alterations and sister-chromatid exchanges in EAC-transplanted murines. Our study further showed that ALE treatment was able to limit liver metallothionein expression, a potential marker for cell proliferation and increased the mean survival of animals to a significant extent. Results indicate that *A ilicifolius* could be used as a potential chemoprotector against neoplasia.

Applications

This study opens up a promising avenue in cancer chemoprevention with indigenous plants. Lack of toxicity favours further preclinical evaluation of *A ilicifolius* in a defined chemical carcinogenesis model. Elucidation of its mechanisms of action at the intricate molecular circuits, and isolation and characterization of the active principles, will provide a better understanding of the treatment strategy, and we would have the beginning of a new chemoprevention programme that could have a broader implication for the well-being of the society.

Peer review

In this experimental study, the authors showed the importance of *Acanthus ilicifolius* plant extract as chemoprotector on EAC-bearing murine model.

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RAPID COMMUNICATION

Choice of laxatives and colonoscopic preparation in pregnant patients from the viewpoint of obstetricians and gastroenterologists

Jeevan Vinod, Jennifer Bonheur, Burton I Korelitz, Georgia Panagopoulos

Jeevan Vinod, Jennifer Bonheur, Burton I Korelitz, Georgia Panagopoulos, Department of Gastroenterology, Lenox Hill Hospital, New York University School of Medicine, New York 10021, United States
Correspondence to: Burton I Korelitz, MD, Department of Gastroenterology, Lenox Hill Hospital, and New York University School of Medicine, 100 East 77th Street, New York, NY 10021, 6 Black Hall, New York, United States. bkorelitz@lenoxhill.net
Telephone: +1-212-4342000
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Abstract

AIM: To elucidate the preferences of gastroenterologists at our institution and compare them to those of obstetricians when making decisions in the pregnant patient, including which type of bowel preparations to use for flexible sigmoidoscopy or colonoscopy, as well as which laxatives can be used safely.

METHODS: Surveys were mailed to all attending gastroenterologists (n = 53) and obstetricians (n = 99) at our institution. Each survey consisted of the 14 most common laxative or motility agents used in pregnancy and inquired about the physician's prescribing habits in the past as well as their willingness to prescribe each medication in the future. The survey also listed four common bowel preparations used prior to colonoscopy and sigmoidoscopy and asked the physician to rank the order of the preferred agent in each case.

RESULTS: With regard to common laxatives, both gastroenterologists and obstetricians favor the use of Metamucil, Colace, and Citrucel. Both groups appear to refrain from using Fleets Phosphosoda and Castor oil. Of note, obstetricians are less inclined to use PEG solution and Miralax, which is not the case with gastroenterologists. In terms of comparing bowel preparations for colonoscopy, 50% of gastroenterologists prefer to use PEG solution and 50% avoid the use of Fleets Phosphosoda. Obstetricians seem to prefer Fleets Phosphosoda (20%) and tend to avoid the use of PEG solution (26%). With regard to bowel preparation for sigmoidoscopy, both groups prefer Fleets enema the most (51%), while magnesium citrate is used least often (38%).

CONCLUSION: It is clear that preferences in the use of bowel cleansing preparations between the two groups exist, but there have not been many case controlled human studies in the pregnant patient that give clear cut indications for using one *versus* another drug. In light of the challenge of performing controlled trials in pregnant women, more extensive surveys should be undertaken to gather a larger amount of data on physicians' experiences and individual preferences.

Key words: Laxative; Pregnancy; Colonoscopy; Sigmoidoscopy; Gastroenterologists; Obstetricians

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INTRODUCTION

There is a degree of apprehension when it comes to altering medical care in pregnant female patients. On some occasions pregnant women will require colonoscopic evaluation and the clinician will be faced with a decision on what type of bowel preparation to use for flexible sigmoidoscopy or colonoscopy. Also, constipation is a common problem faced by many women during the course of pregnancy and a choice of laxatives must be made by the physician. Given the numerous pharmacological choices available and the lack of data in the medical literature, these decisions are not always easy to make.

When considering endoscopy in the pregnant patient, the physician must balance the safety concerns of both mother and fetus against the relative value of the information obtained or the diagnostic procedure performed[1]. All available evidence suggests that sigmoidoscopy is safe during pregnancy and the indications include rectal bleeding, chronic diarrhea, abdominal pain, and rectal pain[2]. Guidelines for colonoscopy in pregnancy are not readily available due to insufficient data, although studies which have been done demonstrate safety and efficacy of the procedure provided that obstetrical consultation and close monitoring take place. Colonoscopy

is indicated for suspected colon cancer, uncontrolled severe hemorrhage, or when necessary before colonic surgery[3] in pregnant women as well as the general population.

After the indications for an endoscopic procedure are established, the next question becomes which bowel preparation to use. Developing guidelines for this poses unique challenges since there are few controlled trials that include pregnant patients. However, there is some information available regarding the efficacy of agents such as tap water enemas, castor oil, polyethylene glycol (PEG) solution, magnesium citrate, senna, bisacodyl, docusate, and phosphosoda[4,5]. The purpose of this study was to elucidate the preferences of gastroenterologists at our institution and compare them to those of obstetricians when making such decisions.

MATERIALS AND METHODS

After obtaining IRB approval, surveys were mailed to all attending gastroenterologists (n = 53) and obstetricians (n = 99) at our institution. Each survey consisted of the 14 most common laxative or motility agents used in pregnancy and inquired about the physician's prescribing habits in the past as well as their willingness to prescribe each medication in the future. The survey also listed four common bowel preparations used prior to colonoscopy and sigmoidoscopy and asked the physician to rank the order of the preferred agent in each case. Descriptive statistics were computed using SPSS Version 14.0. (SPSS Inc. Chicago, Illinois). Valid percentages were used in order to account for those surveys that were incomplete. Utilizing Fisher's Exact Test, the proportion of gastroenterologists who would prescribe the listed laxatives was compared to the proportion of obstetricians who also indicated they would prescribe. $P < 0.5$ was *a priori* considered to indicate statistical significance.

RESULTS

Seventy five surveys were returned making the total response rate nearly 50% (53% of gastroenterologists and 47% of obstetricians). Among gastroenterologists, 96% of respondents were male and 4% female, while 55% of obstetricians were male and 45% female. The mean number of years in practice between both groups was 19 ± 11 years and the mean physician age was 41 ± 22 years.

With regard to the common laxatives, the overwhelming majority of gastroenterologists were inclined to prescribe Metamucil (psyllium), Citrucel (methylcellulose), and Colace (docusate). Some had a preference for using glycerin suppositories, Fleets enemas (sodium phosphate enemas), tap water enemas, and Miralax (PEG 3350), although not as often as the agents above. Meanwhile, most gastroenterologists are reluctant to prescribe Fleets Phosphosoda (oral sodium phosphate), castor oil, and oral Dulcolax (bisacodyl). Table 1 presents the exact percentages as described above.

The vast majority of obstetricians favor the use of Colace, Metamucil, and glycerin suppositories. They also prefer to use Citrucel, Fleets enemas, Dulcolax suppositories, and tap water enemas, although not as often as those listed above. The majority of obstetricians are less inclined to use PEG solution, Mineral oil, and Fleets Phosphosoda. Table 1 presents the exact percentages as described above.

As seen in Table 1, it is evident that both gastroenterologists and obstetricians favor the use of Metamucil (P = 1.00), Colace (P = 0.293), and Citrucel (P = 0.164). Both groups appear to refrain from using Fleets phosphosoda (P = 0.051) and Castor oil (P = 0.402). Of note, obstetricians are less inclined to use PEG solution (P = 0.001), Miralax (P = 0.000), and tap water enema (P = 0.046), which is not the case with gastroenterologists.

In terms of comparing bowel preparations for colonoscopy, it is apparent that 50% of gastroenterologists prefer to use PEG solution and 50% avoid the use of Fleets Phosphosoda. Obstetricians seem to prefer Fleets Phosphosoda (20%) and tend to avoid the use of PEG solution (26%), which is the exact opposite as gastroenterologists. With regard to bowel preparation for sigmoidoscopy, the preferences between gastroenterologists and obstetricians appear to be quite similar. Both groups prefer Fleets enema the most (51%), while magnesium citrate is used least often (38%).

DISCUSSION

Several conclusions can be drawn from the results of this physician survey. Both groups commonly prescribe Metamucil, Citrucel, and Colace. All are frequently used bulk-producing medications that carry low pregnancy risk factors and are without reported complications[6]. The overall frequency of PEG solution being used is much higher with gastroenterologists. Studies have shown that the systemic absorption of PEG is minimal and the problems with abdominal bloating and gas are less common as compared to other osmotic laxatives[5]. Another similarity common to both groups is the avoidance of phosphosoda preparations. This may be related to the fact that in published studies, newborns were shown to manifest bone demineralization and bone growth failure because of maternal phosphate overload[10], although a one time use in pregnancy has not shown to be detrimental. Another consideration when using phosphosoda preparations is the risk of phosphate nephropathy[12], which has been reported in select cases.

The overall majority of the available laxatives are without documented side effects; however castor oil carries an absolute contraindication for use in pregnancy (risk factor X) because it has been linked to uterine rupture[7]. Mineral Oil has also been associated with adverse effects during pregnancy and should be avoided because it can impair maternal fat soluble vitamin absorption, leading to neonatal coagulopathy and hemorrhage[8]. From the results above it appears to be generally avoided in both groups, but significantly more so among obstetricians. Another apparent difference is the prescribing of tap water enemas in both groups. Obstetricians seem more reluctant to prescribe it perhaps due to the possible induction of labor

Table 1 Comparison of gastroenterologists' (GI) *vs* obstetricians' (OB) preference of 14 common laxative/motility agents prescribed in the pregnant patient

	% of GI physicians who have prescribed in the past or would prescribe in the future	% of OB physicians who have prescribed in the past or would prescribe in future	% of GI physicians who would NOT prescribe	% of OB physicians who would NOT prescribe	*P* value
Metamucil (psyllium)	96.5	95.6	3.9	2.2	1.000
Citrucel (methylcellulose)	92.9	77.1	7.1	11.4	0.164
Colace (docusate)	89.3	97.7	10.7	2.3	0.293
Glycerin suppository	77.8	82.0	14.8	15.4	0.757
Fleets enema (sodium phosphate enema)	75.0	55.0	25.0	41.7	0.174
Tap water enema	70.4	44.5	22.2	50.0	0.046
Miralax (PEG 3350)	69.2	20.6	23.1	51.7	0.000
Magnesium Citrate	54.2	32.3	45.8	61.8	0.113
GoLytely (PEG solution)	53.8	12.9	46.2	80.6	0.001
Dulcolax (bisacodyl suppository)	50.0	45.8	34.6	54.3	0.799
Mineral Oil	46.2	25.0	42.3	75.0	0.106
Dulcolax (bisacodyl oral)	30.7	38.3	53.8	52.9	0.595
Castor Oil	26.1	37.6	60.9	62.5	0.402
Fleets Phosphosoda (oral sodium phosphate)	7.7	29.1	92.3	71.0	0.051

with the use of a rectal enema, although it has been shown to be quite safe in the pregnant patient[3].

Another explanation for the differences between the two groups may also be due to preexisting medical conditions in the pregnant patient such as Crohn's disease, which is exacerbated by various medications seen more frequently by gastroenterologists. Such exacerbations may adversely affect the pregnancy[11], although there is no established link with the 14 common laxatives to Crohn's disease exacerbation.

Regarding preparation for colonoscopy, gastroenterologists favor PEG solution, while there appears to be more hesitation among obstetricians. It is unclear whether this is because gastroenterologists have greater experience in its use or simply because data is lacking regarding the safety of medications for colonoscopy and pregnancy. For flexible sigmoidoscopies, both groups favor Fleets enemas, although it has been shown that tap water enemas appear to be the safest option, even if not as effective, particularly during the first trimester[9].

In conclusion, it is clear that preferences in the use of bowel cleansing preparations between the two groups exist, but there have not been many case controlled human studies in the pregnant patient that give clear cut indications for using one *versus* another drug. Therefore, it seems logical that both groups favor medications that seem to respond best with the majority of patients without causing harm or compromising the pregnancy. In light of the challenge of performing controlled trials in pregnant women, more extensive surveys should be undertaken to gather a larger amount of data on physicians' experiences and individual preferences. Perhaps this will provide a clearer understanding of which laxatives and purgatives are optimal to use during pregnancy.

COMMENTS

Background

Prescribing medications to the pregnant patient can be challenging. The purpose of the research study was to gain an understanding of the different practices used by obstetricians and gastroenterologists when prescribing laxatives and using bowel preparations for colonoscopy and sigmoidoscopy.

Research frontiers

There have not been many case controlled human studies in the pregnant patient, therefore surveys were used to gather data on physician prescribing habits.

Innovations and breakthroughs

Due to limited research in the pregnant patient, the majority of articles point toward individual physician preference and patient response when prescribing a particular medication.

Applications

In light of the challenge of performing controlled trials in the pregnant patient, more extensive surveys should be undertaken to gather a larger amount of data on physicians' experiences and individual preferences.

Peer review

This paper is well written and covers an important clinical area. This is an interesting manuscript on the preference of laxatives and colonoscopic preparation in pregnancy of obstetricians and gastroenterologists.

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RAPID COMMUNICATION

Activity and safety of pegylated liposomal doxorubicin, 5-fluorouracil and folinic acid in inoperable hepatocellular carcinoma: A phase Ⅱ study

Giuseppe Di Lorenzo, Antonio Rea, Chiara Carlomagno, Stefano Pepe, Giovannella Palmieri, Roberto Labianca, Antonio Chirianni, Alfonso De Stefano, Vincenzo Esposito, Sabino De Placido, Vincenzo Montesarchio

Giuseppe Di Lorenzo, Chiara Carlomagno, Stefano Pepe, Giovannella Palmieri, Alfonso De Stefano, Sabino De Placido, Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Università degli Studi Federico Ⅱ, Naples, Italy
Antonio Rea, Vincenzo Montesarchio, Divisione di Oncologia, Azienda Ospedaliera Cotugno, Naples, Italy
Antonio Chirianni, Vincenzo Esposito, Divisione di Malattie Infettive Azienda Ospedaliera, Cotugno, Napoli, Italy
Roberto Labianca, Oncology Section, Ospedali Riuniti, Bergamo, Italy
Correspondence to: Giuseppe Di Lorenzo, MD, PhD, Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Università degli Studi Federico Ⅱ, Naples, Italy. giuseppedilorenzoncol@hotmail.com
Telephone: +39-81-7462053 Fax: +39-81-7463660
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Abstract

AIM: To improve the results of New therapeutic strategies in hepatocellular carcinoma (HCC). We have conducted a phase Ⅱ study with pegylated liposomal doxorubicin (PLD), 5-fluorouracil (5FU) and folinic acid (FA).

METHODS: Thirty-one patients with hystologically-confirmed, inoperable HCC, received combination chemotherapy with PLD 25 mg/mq on d 1, 5FU 1200 mg/mq in 48 h continuous infusion, and oral FA 30 mg on d 1 and 2 every 3 wk until disease progression or intolerable toxicity.

RESULTS: The median age was 65 years (range 41-82) and 28 patients were hepatitis C virus seropositive (90%). The majority of patients were Child-Pugh Class B (55%). Two patients showed a partial response (PR), and 16 had stable disease (SD). With a median follow-up of 14 mo, the median time to progression of all evaluable patients was 4 mo (95% CI 1.7-7). Median overall survival was 9 mo (95% CI 3-24 mo). After 1 year, 9 of 18 PR/SD patients were alive. Chemotherapy was well tolerated.

CONCLUSION: PLD/FU/FA combination seems capable of achieving durable stabilization of HCC. The manageable toxicity supports a role for combination with other anticancer agents.

Key words: Pegylated liposomal doxorubicin; 5-fluorouracil; Folinic acid; Hepatocellular carcinoma

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INTRODUCTION

Upward trends in incidence or mortality of hepatocellular carcinoma (HCC) have been reported in the United States, Japan and several European countries, including Italy, over the last two or three decades. This increase has been chiefly attributed to the spread of hepatitis C virus (HCV), which occurred earlier in Japan and Southern Europe than in the United States[1].

Surgical resection and liver transplantation are considered the only cures for HCC, but benefit only about 15% of patients[2]. Patients with unresectable or metastatic disease have a median survival of a few months[3].

It is estimated that about 60% of all patients with HCC have underlying chirrosis. In some patients, chirrosis associated with portal hypertension and thrombocytopenia makes systemic chemotherapy extremely difficult and contributes to the poor prognosis associated with HCC[4].

Free doxorubicin has been shown to be superior to symptomatic treatment of HCC[5], although a meta-analysis has revealed no efficacy of free doxorubicin for treatment of HCC[6]. Pegylated liposomal doxorubicin (PLD) is a novel formulation with markedly delayed clearance, decreased volume of distribution, and accumulation of the drug in malignant tissues, which results in both higher therapeutic efficacy and reduced toxicity[7]. A number of studies have shown, at best, response rates of 10%-17%, with different schedules and a well-tolerated profile[8-10].

5-Fluorouracil (5FU) has demonstrated a modest response rate in HCC[11]. There are no published studies

with combination of PLD and 5FU in HCC. Also, if both drugs show poor activity when used alone, it can be expected that activity will be better when they are used in combination.

We planned a phase Ⅱ study of PLD, 5FU and FA in patients with inoperable HCC. Our schedule was based on a previous phase I study in upper gastrointestinal cancer[12].

MATERIALS AND METHODS

Patients

Patients who were referred to two Italian Departments of Medical Oncology (Cotugno Hospital and Federico Ⅱ University, Naples) because of their metastatic and/or recurrent HCC were considered eligible for study entry. Main eligibility criteria are shown in Table 1. Patients were required to have histologically confirmed HCC, WHO Performance Status < 2, Stage Ⅲ or Ⅳ, Child-Pugh class A or B, and no prior systemic chemotherapy. Other inclusion criteria included an absolute neutrophil count of at least 1500 cells per cubic millimeter and/or at least 100 000 cells per cubic millimeter, adequate renal (serum creatinine level < 2 mg/dL) and cardiac (left ventricular ejection fraction > 50% by echocardiography) function, and signed written informed consent. The study protocol was reviewed and approved by institutional review board.

Treatment

Patients receive PLD (Caelyx; Schering-Plough, Milan, Italy) 25 mg/mq in 500 mL 5% dextrose i.v. over 1 h on d 1. 5FU (Teva Pharma Italia S.r.L., Milan, Italy) 1200 mg/mq was administered for 48 h continuous infusion, and oral FA (Folaren; Ist. Chim. Inter., Rende, Rome, Italy) 30 mg d 1 and 2. Each cycle was given every 3 wk. All patients received a standard supportive regimen consisting of hydration and antiemetics. No prophylactic administration of hematopoietic growth factors was allowed. Treatment was repeated until disease progression or unacceptable toxicity. Retreatment occurred upon recovery of platelets to > 100 000 cells per cubic millimeter, neutrophil count of at least 1500 cells per cubic millimeter and skin toxicity or stomatitis to grade ⩽ 1. A 25% dose reduction was permitted for delayed recovery from toxicity of grade ⩾ 2. Patients were withdrawn from the study if grade 3-4 hematologic toxicity persisted for more than 2 wk.

Response and toxicity evaluation

Baseline examinations were: complete history and examination, electrocardiography, echocardiography, complete blood count, serum alphafetoprotein, weighted magnetic resonance imaging (MRI) of the liver, abdominal ultrasound, and chest X-ray. If MRI was not feasible, computed tomography was used. Response and toxicity were evaluated every three cycles.

The following WHO criteria were used: (1) a complete response (CR) was defined as the disappearance of all radiological and clinical evidence of tumor, and the absence of all tumor-related symptoms for at least 4 wk; (2) a partial response (PR) was defined as a decrease of ⩾ 50% in the product of the largest perpendicular dimensions of measurable tumors, measured at least 4 wk apart; (3) stable disease (SD) was defined as no significant change in radiologic tumor measurements, without worsening in performance status; and (4) progressive disease (PD) was defined as a decline in performance status, the appearance of new malignant lesions, and an increase of ⩾ 25% in measurable disease. Toxicity was based on the National Cancer Institute Common Toxicity Criteria version 2.

Table 1 Main eligibility criteria

Age < 85 yr
WHO performance status 0 or 1
Histologically confirmed HCC
Stage Ⅲ-Ⅳ
No prior systemic chemotherapy
Child-Pugh A or B
Complete history of the patient (including previous treatment)
Informed consent
Normal hematologic, renal and cardiac functions

Statistical analysis

The primary end-point was the response rate and the secondary measures were survival and time to progression. Sample size was calculated to reject a 10% response rate in favor of a target response rate of 30%, with a significance level of 0.05 and a power of 80% by using Simon's optimal two-stage design[13]. In the initial stage, a total of 10 evaluable patients were entered and evaluated for response. If more than one response were observed in the first stage, then 21 additional patients were entered in the second stage to achieve a sample size of 31 evaluable patients. Survival rates and time to progression were assessed by the Kaplan-Meier method. All P values were two-sided, with $P < 0.05$ indicating statistical significance.

RESULTS

A total of 31 patients were included in the study between March 2004 and December 2006. Patient characteristics are summarized in Table 2. The median age was 65 years (range 41-82), and 14 and 17 patients were Child-Pugh class A and B, respectively. The majority of patients (20) had received prior antitumor therapy. The most common previous treatment for HCC was transarterial chemoembolization, which was received by 15 of 31 patients. Twenty-eight patients were HCV seropositive and 22 showed an alphafetoprotein (AFP) level at the upper limit of normal. Fifteen and 16 patients were in stage ⅢC and Ⅳ, respectively, according to the TNM system.

Treatment outcomes

The results are shown in Table 3. Two PR were obtained, and 16 had SD. Sixteen and 8 patients received 6 and 8 treatment cycles, respectively, while 2 were treated with 10 cycles. Among the 16 patients with SD, we observed 8 each in Child-Pugh A and B class. No differences in PR/SD were noted between patients who were pretreated

Table 2 Patient characteristics

No. of patients	31
Male	26
Female	5
Median age (range)	65 (41-82) yr
WHO performance status	
0	12
1	19
Positive hepatitis status	
Hepatitis B	3
Hepatitis C	28
AFP > ULN	
Yes	22
No	9
Child–Pugh status	
A	14
B	17
Disease stage at study entry (TNM)	
Ⅲ C	15
Ⅳ	16
Grading (AJCC) at initial diagnosis	
Well-differentiated	15
Moderately-well-differentiated	10
Poorly differentiated	6
Prior treatment	
Chemoembolization	15
Local alcholization	6
Local radiofrequency	5
None	10

Data are expressed as median (range) or *n*. WHO: World Health Organization. ULN: Upper limit of normal; AJCC: American Joint Committee on Cancer.

Table 3 Responses and survival rates (*n* = 31)

Response after three cycles	No. of patients (%)
PR	2 (6.5)
SD	16 (51.5)
Progression	13 (42)
Median progression-free survival (95% CI)	4 (1.7-7) mo
Overall survival rate	
6 mo	21 (68)
12 mo	9 (29)
24 mo	1 (0.3)
Survival rate in PR or SD patients (*n* = 18)	
6 mo	15 (83)
12 mo	9 (50)
24 mo	1 (0.56)

Table 4 Reported toxicity by 31 patients *n* (%)

Toxicity	Grade 1-2	Grade 3-4
Neutropenia	7 (22.5)	3 (10)
Anemia	8 (26)	2 (6.5)
Thrombocytopenia	5 (16)	2 (6.5)
Oral stomatitis	7 (22.5)	1 (3)
Nausea/vomiting	3 (10)	2 (6.5)
Diarrhea	3 (10)	1 (3)
Hand-foot syndrome	5 (16)	0
Hepatic dysfunction	3 (10)	0
Peripheral neuropathy	3 (10)	0

and those who were not, such as those in stage ⅢC and Ⅳ. With a median follow-up of 14 mo, the median time to progression of all evaluable patients was 4 mo (95% CI 1.7-7). Median overall survival was 9 mo (95% CI 3-24 mo). Among 18 responder/SD patients, 9 (50%) were alive at 12 mo, while at the same time, none of the PD patients were alive ($P < 0.05$). One responder who received 10 cycles of chemotherapy was alive at 24 mo. Of 31 evaluable patients, 16 (52%) showed a > 50% decrease in serum AFP from baseline. Among 13 patients with PD, 5 progressed to Child-Pugh C class. After disease progression, 6 patients had salvage treatment with platinum compound or thalidomide, while 25 patients received only supportive care.

Safety

In total, 130 cycles of chemotherapy were administered, with a median of 6 cycles (range 2-10), and 13 of the planned cycles were delayed because of toxic effects. Dose reduction was required in 20 cycles. All eligible patients were evaluable for toxic effects (Table 4). The most frequent toxic effects were neutropenia grade 1-2 and oral stomatitis in 7 patients each. These effects were well managed and did not require treatment discontinuation. Grade 3-4 anemia, neutropenia and thrombocytopenia were observed in 2, 3 and 2 patients, respectively. These side effects required hematopoietic growth factors and red-cell transfusion during the treatment. Hand-foot skin rash occurred in 5 patients and was limited to low-grade intensity.

DISCUSSION

There is no consensus on the treatment of patients with advanced-stage HCC. Locoregional approaches (embolization, intra-arterial chemotherapy and chemoembolization) and systemic treatments have offered marginal survival benefit in most clinical trials[14]. The overall response rate to systemic chemotherapy is generally < 10%[15], probably owing to the strong multidrug-resistance gene expression that is usually observed in this setting[16]. Doxorubicin has been widely evaluated in patients with advanced stage HCC, either alone or in combination. The combination of cisplatin, doxorubicin, 5FU and alpha-interferon (PIAF) has been assessed in 50 patients. The overall response rate was 26%, but considerable hematological toxicity and 2 treatment-related deaths occurred[17]. There was an expectation that doxorubicin efficacy would be enhanced with the better-tolerated PLD formulation[18].

A number of studies have shown, with different doses and schedules, at best, response rates of 10%-17% with PLD alone[8-10]. Two other studies have revealed no response, and with a median survival of 20 wk, the authors concluded that PLD was ineffective[19,20]. Based on a median survival of 1 year, Schmidinger *et al*[9] concluded that PLD warrented further study; however, Ruff *et al*[8] achieved a median survival of only 5.3 mo.

5-fluorouracil has been used alone in HCC, with modest activity[11]. In an attempt to achieve better effectiveness we have combined PLD and 5FU. To date, there have been no published studies with PLD and

5FU in HCC. We chose to use PLD 25 mg/mq and 5FU 1200 mg/mq every 3 wk since we considered, as a result of a previous phase I study[12], that these doses would be well tolerated. In our trial, the combination of PLD, 5FU and FA was well tolerated, and we achieved an overall response rate of 6.5%. However, despite the modest overall response rate, we observed SD in > 50% of patients. This information is encouraging, and PLD/FU/FA combination seems capable of achieving durable stabilization of HCC.

Median time to progression was 4 mo, which is longer than that in other PLD studies, such as those by Valle *et al* and Halm *et al*[19,21]. Median overall survival was 9 mo and was shorter than that in the study by Schmidinger *et al*[9], but longer than that in previous PLD studies[8,10,19,21].

Among 18 responder/SD patients, nine (50%) were alive at 12 mo, while at the same time, none of those with PD was alive ($P < 0.05$). Our SD rate (50%) was higher than that in the studies by Valle *et al* and Halm *et al*. Our study showed better results than those in a recent study by Poh *et al,* which showed no objective response after treatment with combined PLD and 5FU analogue (capecitabine)[22]. Higher PR and SD rates, and longer survival were observed by Louafi *et al*[23]. Thirty-four patients have been treated with gemcitabine plus oxaliplatin (GEMOX). The overall response rate was 18% and SD was observed in 58% of patients. Median overall survival was 11.5 mo.

However, these differences could have been due to different selection criteria. The GEMOX trial included few Child-Pugh Class B and pretreated patients compared to our study. It should be kept in mind that the degree of cirrhosis in our patients was not only limited to Child-Pugh class A but also Class B patients. In our trial, the majority of patients were Class B. Other studies have not included Class B patients[24], have not indicated whether they include Class B patients[21], or if this class was included, it was in the minority[19,23]. This point strengthens our results. In the past 15 years, about 10 new chemotherapeutic agents have become available, which has led to major advances in the medical treatment of several gastrointestinal tract cancers, such as colon adenocarcinoma. However, recent clinical trials of the taxanes irinotecan and topotecan have all given disappointing results for HCC[25-27]. New targeted antitumoral therapies have been tested in HCC patients over the past 2 years. Preliminary results of a phase Ⅲ randomized trial have shown that first-line treatment with sorafenib improves survival in HCC when compared to placebo[28].

In conclusion, a major limit of our study was that it was a small phase Ⅱ study; however, we believe that the results, especially in terms of long-term disease stabilization, merit further evaluation. For example, it would be interesting to carry out additional clinical studies that involve novel targeted agents in combination with cytotoxic agents such as PLD and FUFA. One of the next steps will be to combine this schedule with biological agents in HCC, such as bortezomib, since synergy between bortezomib and anthracyclines has been observed[29].

COMMENTS

Background

There is no consensus on the treatment of patients with advanced-stage HCC. Locoregional approaches (embolization, intra-arterial chemotherapy and chemoembolization) and systemic treatments have offered a marginal survival benefit in most clinical trials.

Research frontiers

The overall response rate to systemic chemotherapy is generally < 10%, probably owing to the strong multidrug-resistance gene expression that is usually observed in this setting.

Related publications

A number of studies have shown, with different doses and schedules, at best, response rates of 10%-17% with PLD alone. 5FU has been used alone in HCC, with modest activity.

Innovations and breakthroughs

In an attempt to achieve better effectiveness, we combined PLD and 5FU. To date, there have been no published studies with PLD and 5FU in HCC. In our trial, combination of PLD, 5FU and FA was well tolerated, and we achieved an overall response rate of 6.5%. However, despite the modest overall response rate, we observed SD in > 50% of patients. Our SD rate is higher than that in the studies of Valle *et al* and Halm *et al*. Median time to progression was 4 mo, which is higher than that in other PLD studies.

Applications

It should be kept in mind that the degree of cirrhosis of our patients was not only limited to Child-Pugh class A but also Class B patients. In our trial, the majority of patients were Class B. Other studies did not include Class B patients, did not indicate whether they included Class B patients, or if this class was included, it was in the minority. This point strengthens our results. We believe that our results, especially in terms of long-term disease stabilization, merit further evaluation. It would be interesting to carry out additional clinical studies that involve novel targeted agents, such as bortezomib, in combination with PLD and FUFA.

Terminology

Pegylated liposomal doxorubicin (PLD) is a novel formulation with markedly delayed clearance, decreased volume of distribution, and accumulation of the drug in malignant tissues, which results in both higher therapeutic efficacy and reduced toxicity.

Peer review

Although this study had a small sample size, the results are interesting. Inoperable HCC was stabilized with chemotherapy that was well tolerated. In addition, this is believed to be the first report of the use of PLD in combination with 5FU. As for reviewer comments, how do the authors explain the modest overall response rate of 6.5% and yet stabilization of disease in 51.1%? Was there any progression in the liver disease or hepatic function tests during the administration of chemotherapy? Did any patients progress to Child-Pugh class C?

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RAPID COMMUNICATION

PlexinA1 expression in gastric carcinoma and its relationship with tumor angiogenesis and proliferation

Xiang-Yang Zhao, Lin Chen, Yu-Hong Li, Qian Xu

Xiang-Yang Zhao, Lin Chen, Department of General Surgery, General Hospital of Chinese PLA, Beijing 100853, China
Yu-Hong Li, Department of Pathology, Chengde Medical College, Chende 067000, Hebei Province, China
Qian Xu, Institute of Basic Medicine, Chengde Medical College, Chengde 067000, Hebei Province, China
Supported by The National Natural Science Foundation of China, No 30570522
Correspondence to: Professor Lin Chen, Department of General Surgery, General Hospital of Chinese PLA, 28 Fuxing Road, Beijing 100853, China. chenlinbj@vip.sina.com
Telephone: +86-10-66937846 Fax: +86-10-6818168
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Abstract

AIM: To explore the expression of PlexinA1 in gastric carcinoma and its relationship with tumor angiogenesis and proliferation.

METHODS: PlexinA1 mRNA and protein expressions of Semaphorin6D were measured using semi-quantity reverse transcription PCR and Western blotting in 20 cases of gastric carcinoma and corresponding normal gastric mucosa. PlexinA1, Ki-67 expression and microvessel density (MVD) were detected by immunohistochemistry in 50 cases of gastric carcinoma and 20 cases of normal gastric mucosa.

RESULTS: The mRNA and protein expressions of PlexinA1 in gastric carcinoma were significantly higher than that in normal gastric mucosa (0.71 ± 0.37 *vs* 0.60 ± 0.25, $P = 0.0299 < 0.05$, and 0.47 ± 0.16 vs 0.21 ± 0.08, $P = 0.0000 < 0.01$), and MVD within tumor tissues increased significantly with PlexinA1 mRNA expression ($r = 0.8736$, $P < 0.01$) and PlexinA1 protein expression ($r = 0.7286$, $P < 0.01$), and MVD of the PlexinA1 positive staining group (25.25 ± 3.93) was significantly higher than that of the negative group (19.56 ± 1.75), ($P < 0.01$). Proliferation index of tumor cells within tumor tissues were positively correlated with PlexinA1 mRNA expression ($r = 0.5420$, $P = 0.014 < 0.01$) and PlexinA1 protein expression ($r = 0.5024$, $P = 0.024 < 0.05$). The proliferation index of the PlexinA1 positive staining group (567.69 ± 125.61) was significantly higher than that of the negative group (369.58 ± 116.88), ($P < 0.01$).

CONCLUSION: PlexinA1 may play an important role in the occurrence and development of gastric carcinoma, and be related to tumor angiogenesis and proliferation.

Key words: PlexinA1; Gastric carcinoma; Angiogenesis; Proliferation

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INTRODUCTION

Primary gastric carcinoma is one of the most common malignant tumors in China. Invasion and metastasis are the main causes for the death of cancer patients. On the other hand, invasion and metastasis of malignant tumors are closely related to the proliferation of tumor cells and angiogenesis of tumor tissues. PlexinA1 is a large transmembrane protein that is a major receptor for multiple classes of Somaphorins, either alone or in combination with neuropilins. Although PlexinA1 has pleiotropic function in formation of nervous systems, embryogenesis, angiogenesis and immunoreaction[1-4], the function of Plexin A1 in carcinogenesis remain unrevealed. Therefore, we detected the protein and mRNA expression of PlexinA1 in gastric carcinoma and normal gastric mucosa by the semi-quantitative RT-PCR, Western blotting and immunohistochemistry in order to explore the expression of PlexinA1 in gastric carcinoma and its relationship with tumor angiogenesis and proliferation.

MATERIALS AND METHODS

Patients

Twenty fresh gastric carcinoma specimens and corresponding normal gastric mucosa specimens were analyzed by RT-PCR and Western blotting. All specimens were obtained from patients (8 women and 12 man; mean age 54 years) who underwent surgery for gastric carcinoma between April 2006 and August 2006 in the General Hospital of PLA. Of the 20 patients, 9 showed high-moderate differentiation, 11 poor differentiation and 10 lymph node metastasis. Fifty paraffin-embedded gastric carcinoma specimens and twenty normal gastric mucosa specimens were collected respectively for immunohistochemistry. The patients with gastric carcinoma consisted of 21 women and 29 men with a mean age of 56 years. Of the 50 gastric carcinoma specimens, 23 were highly-moderately differentiated and

27 poorly differented, and 28 had lymph node metastasis. The diagnosis was confirmed by pathological examination.

Reverse transcription PCR (RT-PCR)

Total mRNA was isolated by Trizol reagent according to the procedure of the supplier (BioDev-tech, Beijing, China), and the concentration was determined by measuring the absorbance at 260 nm and using the following data???: 1 optical density unit = 40 mg of RNA/mL. A 1.5 μg aliquot of total RNA from each specimen was reverse-transcribed into single-strand cDNA using oligo (dT)16 primer for 2 h at 37℃, each single-strand cDNA was used for subsequent PCR amplification of PlexinA1 and β-actin with the latter used as a quantitative control. The PCR was carried out in a reaction volume of 50 μL for 5 min at 95℃ for initial denaturing, followed by 37 cycles of 94℃ for 50 s, 55℃ for 50 s, and 72℃ for 1 min, then extend at 72℃ for 10 min on the Authorized Thermal Cycler for PCR. The primer sequences used for amplification were 5'-TGTGGACGACCCCAAATT CTA-3' and 5'-CTGGGCAAACACGGTGAAC-3' for PlexinA1, 5'-ACACCTACCAGGGAACGGAG-3' and 3' -GCCTCTGCACATACCTGCT-5' for β-actin. The primer sequences were synthesized by Beijing Genomics Institute (China). PCR products were resolved in 2% agarose gels and visualized by staining with ethidium bromide. To quantify PCR products, the bands representing amplified products were analyzed by Quantity One Analysis Software (BIO-RAD Co. America).

Western blotting

Expression of the PlexinA1 protein was detected using the Western blot method. After washing in ice-cold PBS, the samples were finely minced and suspended in ice-cold homogenization buffer (2 mL/g tissue), which contained protease inhibitors to minimize protein degradation. The suspension was firstly homogenized, then centrifuged at 12000 × *g* for 30 min at 4℃ to remove the nuclei and cell debris. The supernatant (total protein extract) was collected. Equal amount (50 μg) of proteins was run on a 10% SDS-PAGE gel and electrotransferred onto Hybond-polyvinylidene difluoride membranes (Amersham, Arlington Heights, USA). The membranes were blocked for 2 h at room temperature, followed by incubation with the primary anti-PlexinA1 antibody 1:50 (Santa Cruz Co, USA) at 4℃ overnight. The primary antibody was diluted in TBST containing fat-free milk. After three 10-min washes in TBST, the membrane was incubated in peroxidase-conjugated secondary antibody (Sigma, St. Louis, USA) diluted 1:800 at room temperature for 1 h. Immunoreactive proteins were visualized by autoradiogram using ECL Western blotting detection reagents (Amershan Pharmacia, Uppsala, Sweden) and exposing to X-Omat BT film (Kodak, New York, USA). Bands were analyzed by Quantity One Analysis Software

Immunohistochemistry

PlexinA1 multiclonal antibody 1:50 was purchased from American Santa Cruz Co. Monoclonal antibody of factor Ⅷ and monoclonal antibody of Ki-67 (ready to use) were supplied by the Chinese Boster Co. All operations were performed according to the instructions of the manufacturers. Positive specimens were used as positive controls and PBS in substitution of the first antibody was used as a negative control at the same time.

PlexinA1 expression

The cytoplasm and membrane of positive cells were stained brown by PlexinA1. The result of immunostaining was recorded as negative or positive based on the expression of protein detected. At least 10 surface areas were scored, and the percentage of positive cells was calculated for each specimen. Specimens were classified as positive if more than 30% of the cells were stained at × 400 magnification.

Proliferation index of tumor cells

The nuclei of positive cells were stained deep brown by Ki-67. The number of positive cells among 1000 tumor cells was counted per slide and taken as the tumor cell proliferation index.

MVD of gastric carcinoma tissues

Gastric carcinoma vascular endothelial cells were stained brown. The isolated brown and yellow blood vessel endothelial cells or cell clusters in gastric carcinoma tissues were regarded as a single microvessel (Figure 1A). Areas with the highest microvessel densities were selected under × 100 microscopic magnification. Then the number of microvessels stained by facto Ⅷ antibody in 5 vision fields was counted under × 400 magnification, respectively. The average values were taken as MVD[5]. Indistinguishable or indistinct cells were excluded.

Statistical analysis

All data were analyzed by SPSS12.0 statistical software. *t* test, analysis of Chi-square test, and linear correlation analysis were used. $P < 0.05$ was taken as significant.

RESULTS

Expression of PlexinA1 mRNA by RT-PCR

Two percent of Agarose gel electrophoresis showed a 172 bp PlexinA1 fragment by RT-PCR amplification from gastric caner specimens and normal gastric mucosa (Figure 2). The PlexinA1 mRNA amplification was successful in all tissues. The expression level was higher in tumor (0.71 ± 0.37) than in normal gastric mucosa (0.60 ± 0.25, $P = 0.0299 < 0.05$).

Expression of PlexinA1 protein by western blotting

The affinity-purified anti-plexinA1 antibody detected a major band at 90 kD in protein extracts from all samples tested (Figure 3). The expression level was much higher in tumor (0.47 ± 0.16) than that in normal gastric mucosa (0.21 ± 0.08, $P < 0.01$). This result is matched with that of RT-PCR.

Expression of PlexinA1 by immunohistochemistry

In immunohistochemical staining, PlexinA1 located at the membrane and cytoplasm of gastric carcinoma cells appeared as brown particles. The positive expression rate of PlexinA1 in gastric carcinoma was 52%, significantly

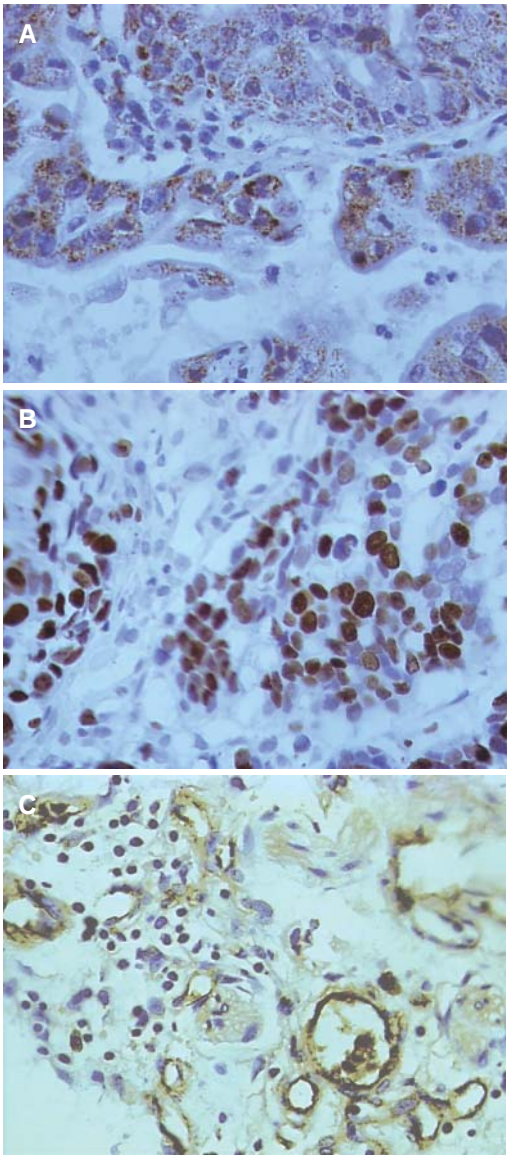

Figure 1 Expression of PlexinA1, Ki-67 and Ⅷ factor in gastric carcinoma tissue (S-P × 400). **A**: PlexinA1; **B**: Ki-67; **C**: Ⅷ factor.

higher than that in normal gastric mucosa (25%), ($P < 0.05$. However, the PlexinA1 expression level had no correlation with the age of patients, tumor size, invasion depth, differentiation degree and lymph node metastasis (Table 1).

Correlation between PlexinA1 and proliferation index of tumor cells in gastric carcinoma

PlexinA1 mRNA and protein expression ratio of gastric carcinoma and the corresponding normal gastric mucosa (T/N) was significantly correlated with proliferation index of tumor cells in gastric carcinoma. Proliferation index of tumor cells within tumorous tissues increased significantly with PlexinA1 mRNA expression ($r = 0.5420$, $P = 0.014 < 0.05$) and PlexinA1 protein expression ($r = 0.5024$, $P = 0.024 < 0.05$). The proliferation index of the PlexinA1 positive staining group (567.69 ± 125.61) was significantly higher than that of the negative group (369.58±116.88), ($P < 0.01$).

PlexinA1 expression and the MVD of tumor tissues

PlexinA1 mRNA and protein expression ratio of gastric carcinoma and the corresponding normal gastric mucosa (T/N) was significantly correlated with the MVD of gastric carcinoma. MVD was elevated with the increase of PlexinA1 mRNA and protein expression. MVD within tumorous tissues increased significantly with PlexinA1 mRNA expression ($r = 0.8736$, $P < 0.01$) and PlexinA1 protein expression ($r = 0.7286$, $P < 0.01$). Also, with increase of PlexinA1 expression of gastric carcinoma tissues by immunohistochemistry, MVD rose as well. The MVD of the PlexinA1 positive staining group (25.25 ± 3.93) was significantly higher than that of the negative group (19.56 ± 1.75), ($P < 0.01$).

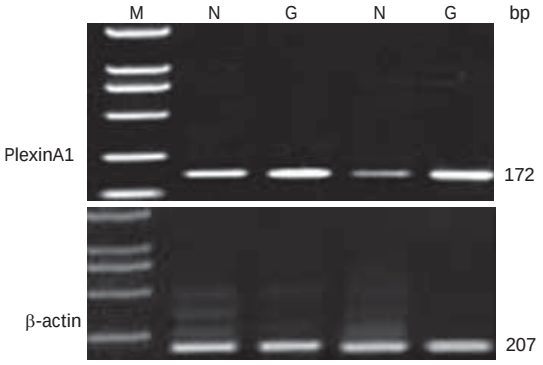

Figure 2 RT-PCR was performed to detect the expressions of PlexinA1 mRNA. M: Marker; N: Normal gastric mucosa; G: Gastric carcinoma.

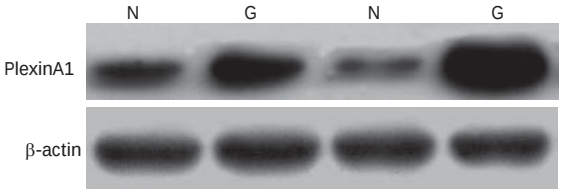

Figure 3 Western Blotting was performed to detect the protein expressions of PlexinA1. N: Normal gastric mucosa; G: Gastric carcinoma.

Table 1 The relationship between PlexinA1 expression and clinicopathologicl characteristics in gastric carcinoma

Characteristics	PlexinA1		Total	*P* value
	Negative	Positive		
Age (yr)				
⩾ 45	12	14	26	> 0.05
< 45	12	12	24	
Size				
⩾ 5 cm	8	11	19	> 0.05
< 5 cm	16	15	31	
Differentiation				
Well-moderate	13	10	23	> 0.05
Poorly	11	16	27	
Invasion depth				
Lamina and muscularis propria	5	6	11	> 0.05
Visceral peritoneum	19	20	39	
Lymph node metatasis				
Negative	10	12	22	> 0.05
Positive	14	14	28	

DISCUSSION

PlexinA1 is a large transmembrane protein that is receptor

of Semaphorins, either alone or in combination with neuropilin-1 or -2[1,6]. Despite the fact that PlexinA1 plays a crucial role in formation of the nervous system, increasing evidence attested to the significance of PlexinA1 in cardiogenesis. Some works reported that PlexinA1 has multiple functions in cardiogenesis as a receptor for the transmembrane Semaphorin, Sema6D, independent of neuropilins, and it plays a critical role in cardiac morphogenesis by regulating epithelial cell migration[2,7]. These findings suggest that PlexinA1 may regulate angiogenesis in vivo, and raise the intriguing possibility that PlexinA1 may play a role in tumor-induced angiogenesis.

As it is well known, the growth and metastasis of tumors require induction of angiogenesis[8,9]. Without the ability to induce angiogenesis, most neoplasms would fail to grow > 2 mm in diameter or metastasize, including gastric carcinoma. We detected the mRNA and protein expression of PlexinA1 in gastric carcinoma and normal gastric mucosa by the semi-quantity RT-PCR and Western blotting. The results showed that the expression of PlexinA1 in gastric carcinoma was significantly higher than those in normal gastric mucosa. We further detected the expression of PlexinA1 in gastric carcinoma and normal gastric mucosa by immunohistochemistry. The positive expression rate of PlexinA1 in gastric carcinoma was significantly higher than that in normal gastric mucosa. However, the PlexinA1 expression level was not correlated with the age of patients, tumor size, invasion depth, differentiation degree, and lymph node metastasis. These results suggest that PlexinA1 may play an important role in the occurrence and development of gastric carcinoma. MVD and Ki-67 are important index of judging tumor angiogenesis and tumor cell proliferation[10-12].

Under the effect of angiogenesis and proliferation factors, tumor and endothelial cells proliferate and migrate to form new blood vessel networks and induce tumor invasion and metastasis. This study showed that MVD within tumor tissues increased significantly with PlexinA1 mRNA and protein expression, and PlexinA1 expression level of cancer tissues was positively correlated with Ki-67. These results suggest that PlexinA1 may contribute to tumor angiogenesis and tumor cell proliferation through binding its ligands named Semaphorins.

In conclusion, this is the first investigation about the expression of PlexinA1 in gastric carcinoma and its clinicopathological significance. The results of our study shed some light on the pathogenesis of gastric carcinoma, and may represent a new therapeutic target for gastric carcinoma treatment.

COMMENTS

Background

Primary gastric carcinoma is one of the most common malignant tumors in China. Invasion and metastasis are the main causes for the death of cancer patients, and invasion and metastasis of malignant tumors are closely related with proliferation of tumor cells and angiogenesis of tumor tissues. PlexinA1 is a large transmembrane protein that is a major receptor for multiple classes of Somaphorins, and has pleiotropic function in formation of nervous systems, embryogenesis, angiogenesis and immunoreaction, yet the function of PlexinA1 in carcinogenesis has not been intensively studied, including in gastric carcinoma.

Research frontiers

Experiments have been employed to study the expression of PlexinA1 in gastric carcinoma and its relationship with tumor angiogenesis and proliferation. These studies show that the expression level in gastric carcinoma is higher than that in normal gastric mucosa, and is positively related to tumor angiogenesis and proliferation.

Innovations and breakthroughs

This is the first investigation about the expression of PlexinA1 in gastric carcinoma and its clinicopathological significance. PlexinA1 is found positively related to tumor angiogenesis and proliferation, which shed some light on the pathogenesis of gastric carcinoma.

Applications

This study may represent a new therapeutic target for gastric carcinoma treatment.

Peer review

The authors studied the expression of PlexinA1 in gastric carcinoma and its relationship with tumor angiogenesis and proliferation, and showed that the expression level in gastric carcinoma is higher than that in normal gastric mucosa, and is positively related to tumor angiogenesis and proliferation, which may be useful in basic research of gastric carcinoma, and provide a new thought about gastric carcinoma treatment.

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RAPID COMMUNICATION

Serum pepsinogen levels and their influencing factors: A population-based study in 6990 Chinese from North China

Li-Ping Sun, Yue-Hua Gong, Lan Wang, Yuan Yuan

Li-Ping Sun, Yue-Hua Gong, Lan Wang, Yuan Yuan, Cancer Institute, First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China
Supported by National Key Technologies R&D Program of China during the 10th Five-year Plan Period, No. 2001BA703B06 (B), 2004BA703B04-02
Correspondence to: Dr. Yuan Yuan, Cancer Institute, First Affiliated Hospital, China Medical University, 155 Nanjing North Street, Shenyang 110001, Liaoning Province, China. yyuan@mail.cmu.edu.cn
Telephone: +86-24-83282153 Fax: +86-24-83282292
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Abstract

AIM: To explore the essential characteristics of serum pepsinogen (PG) levels in Chinese people, by analyzing the population-based data on the serum levels of PG Ⅰ and Ⅱ and the PG Ⅰ/Ⅱ ratio, and their influencing factors in Chinese from North China.

METHODS: A total of 6990 subjects, who underwent a gastric cancer screening in North China from 1997 to 2002, were collected in this study. Serum pepsinogen levels were measured by enzyme-linked immunosorbent assay (ELISA). *H pylori* status was determined by histological examination and *H pylori*-IgG ELISA. The cut-off point was calculated by using receiving operator characteristics (ROC) curves. Factors linked to serum PG Ⅰ/Ⅱ ratio were identified using a multivariate logistic regression.

RESULTS: The serum PG Ⅰ and PG Ⅱ levels were significantly higher in males than in females (95.2 μg/L *vs* 79.7 μg/L, $P < 0.01$; 12.1 μg/L *vs* 9.4 μg/L, $P < 0.01$), PG Ⅰ/Ⅱ ratio was significantly lower in males than in females (7.9 *vs* 8.3, $P < 0.01$). The PG Ⅰ/Ⅱ ratio decreased significantly in the aged groups following the progression of gastric mucosa from normal to non-atrophic and atrophic lesions (10.4, 8.8, and 6.6, respectively). The serum PG Ⅰ and Ⅱ levels were significantly higher in patients with *H pylori* infection than in those without *H pylori* infection (88.7 μg/L *vs* 81.4 μg/L, $P < 0.01$; 11.4 μg/L *vs* 8.4 μg/L, $P < 0.01$), while the PG Ⅰ/Ⅱ ratio was significantly lower in patients with *H pylori* infection than in those without *H pylori* infection (7.7 *vs* 9.6, $P < 0.01$). For patients with atrophic lesions, the area under the PG Ⅰ/Ⅱ ROC curve was 0.622. The best cut-off point for PG Ⅰ/Ⅱ was 6.9, with a sensitivity of 53.2%, and a specificity of 67.5%. Factors linked to PG Ⅰ/Ⅱ were sensitive to identified PG using a multinomial logistic regression relying on the following inputs: males (OR: 1.151, 95% CI: 1.042-1.272, $P = 0.006$), age ⩾ 61 years (OR: 1.358, 95% CI: 1.188-1.553, $P = 0.000$), atrophic lesion (OR: 2.075, 95% CI: 1.870-2.302, $P = 0.000$), and *H pylori* infection (OR: 1.546, 95% CI: 1.368-1.748, $P = 0.000$).

CONCLUSION: The essential characteristics of serum PG levels in Chinese are significantly skewed from the normal distribution, and influenced by age, sex, gastric mucosa lesions and *H pylori* infection. PG Ⅰ/Ⅱ ratio is more suitable for identifying subgroups with different influence factors compared with PG Ⅰ or PG Ⅱ alone.

Key words: Pepsinogen; Gastric cancer; *Helicobacter pylori*; Screening

Sun LP, Gong YH, Wang L, Yuan Y. Serum pepsinogen levels and their influencing factors: A population-based study in 6990 Chinese from North China. *World J Gastroenterol* 2007; 13(48): 6562-6567

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INTRODUCTION

Human pepsinogens (PGs) are inactive pro-enzymes for the specific digestive enzyme--pepsin originating from the gastric mucosa, and can be classified biochemically and immunochemically into pepsinogen Ⅰ (PG Ⅰ) and pepsinogen Ⅱ (PG Ⅱ). Both of them are secreted by the chief and mucous neck cells of the gastric fundus and corpus. PG Ⅱ is also secreted by the pylori glands in the antrum and Brunner's glands in the proximal duodenum. PG Ⅰ and PG Ⅱ are secreted into the gastric lumen and 1% of them are also leaked into circulating blood[1,2]. PG levels in blood seem to be related to the morphologic and functional changes in the stomach, and their use as 'serological biopsy' has been reported over 20 years before[3-5]. Recently, more and more investigators are concerned about the relationship between serum PG levels and gastric precancerous diseases, gastric carcinogenesis, and the significance of them being a marker for the screening of gastric cancer (GC). In most Western countries, the focus has been on the

identification of individuals for intervention studies, whereas in Japan the use of PG levels is to identify those for endoscopic examination and those at risk for GC[6-10]. However, the limited knowledge about their characteristics in different populations and the significant differences in methodologies may prejudice the assessment of consistency. For instance, different cut-off values are used for the positive definition when either PG Ⅰ levels or both PG Ⅰ and PG Ⅱ levels are considered[11-13]. Furthermore, due to few cohort studies have been done in Chinese, the population-based data on serum PG levels and their influencing factors, such as age, sex, the presence of different gastric diseases and *H pylori* infection, are limited[14,15].

In the present study, we measured the serum PG Ⅰ, PG Ⅱ levels in residents from the Zhuanghe County, Liaoning Province, in North China, in order to investigate the essential characteristicss of serum PG levels in Chinese, which is expected to provide a valuable reference for large-scale surveys of gastric cancer.

MATERIALS AND METHODS

Subjects

A total of 6990 subjects (3455 men and 3535 women), who underwent gastric cancer screening in North China from 1997 to 2002, were enrolled in this study. Their mean age was 48.84 years, ranging 11-82 years. Information about gender, age and other factors was obtained by means of a questionnaire administered to each subject. The study was approved by the Human Ethics Review Committee of China Medical University. Written informed consent was obtained from participants in accordance with the Declaration of Helsinki and its later revision.

Serum pepsinogen level

Approximately 5 mL fasting blood was collected from each participant and kept at 4℃ for 24 h. The blood was centrifuged at 3000 r/min for 10 min and the serum aliquot was stored immediately at -20℃ and then shifted to an environment at -70℃ for the determination of various parameters. Serum PG concentration was measured by enzyme-linked immunosorbent assay (ELISA) with PG Ⅰ/PG Ⅱ ELISA kits (Biohit Co., Ltd., Finland).

Endoscopic and clinicopathological examinations

Gastrointestinal endoscopy was performed for observing the entire stomach. Experienced endoscopists performed each examination without knowledge about the serological data on the study subjects. Gastric mucosa was examined, and 4 biopsy specimens were obtained from the body, angulus, antrum and lesions, respectively. The biopsies were routinely bathed in 10% formalin, embedded in paraffin, then sectioned and stained in each local center. The stained sections were evaluated independently by two gastrointestinal pathologists. Each subject was assigned a global diagnosis based on the 4 specimens. Microscopic findings were assessed according to the consensus on chronic gastritis at the national symposium[16] or in combination with the visual analog scale of the updated Sydney System[17], including normal mucosa (NOR, n = 494), superficial gastritis (SG, n = 3954), erosive gastritis and ulcers (GEU, n = 362), superficial gastritis accompanying IM (SG-IM, n = 347), atrophic gastritis (AG, n = 870), gastric polyp(GP, 73 cases), dysplasia (GD, n = 110) and GC (n = 80).

Identification of H pylori infection

Gastric biopsies were evaluated for *H pylori* infection by histological examination. *H pylori* could be found in gastric epithelium or in mucus. Serum immunoglobulin (Ig)G antibodies to *H pylori* were detected by ELISA with *H pylori*-IgG ELISA kit (Biohit Co., Ltd., Finland) in duplicate. Patients whose antibody titer defined by optical density (OD)values according to the manufacturer's protocol, was higher than the cut off value of 42EIU, were regarded as positive for *H pylori* infection.

Statistical analysis

All statistical analyses were performed using SPSS 11.5 software (SPSS Inc. Chicago, USA). The distribution of variables was tested by "Kolmogorov-Smirnov". The relation between two continuous variables was assessed by Spearman's correlation coefficient. The median of variables was compared between two groups by the Mann-Whitney U test and multiple comparisons by the Kruskal-Wallis test. The receiving operating characteristics (ROC) curve for each evaluation was used to extract the corresponding cut-off point, which can be used to discriminate different histological patterns of patients. For that purpose, the area under each ROC curve was used to measure the discriminatory ability of the model. The resulting value of the cut-off point for each evaluation was applied to the determination of the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the test. Consequently, 95% confidence interval was calculated. Different variables influencing the values of PG Ⅰ, PG Ⅱ and PG Ⅰ/Ⅱ ratio were identified with multivariate logistic regressions adjusted by the histological diagnosis. The value was shown as the median. A two-sided P value of less than 0.05 was considered statistically significant.

RESULTS

Basic population data on PG levels in Chinese

Among the 6990 subjects analyzed, the serum levels of PG Ⅰ and PG Ⅱ and the PG Ⅰ/Ⅱ ratio were significantly skewed from the normal distribution. The median value was 86.9 μg/L, 10.6 μg/L and 8.1 μg/L, respectively.

PG levels in different gender groups

The gender was significantly correlated with the serum PG Ⅰ and PG Ⅱ level (r = -0.178, r = -0.147, P = 0.000). The PG Ⅰ and PG Ⅱ levels were significantly higher in males than in females, while the PG Ⅰ/Ⅱ ratio was significantly lower in males than in females (Table 1).

PG levels in different age groups

All the objects were assigned to four groups based on their

Table 1 Serum PG levels in different gender groups (median)

Gender	Cases (*n*)	PG Ⅰ (μg/L)	PG Ⅱ (μg/L)	PG Ⅰ / Ⅱ
Male	3455	95.2	12.1	7.9
Female	3535	79.7	9.4	8.3
P value		0.000	0.000	0.000

Table 2 Serum PG levels in different age groups (median)

Age (yr)	Cases (*n*)	PG Ⅰ (μg/L)	PG Ⅱ (μg/L)	PG Ⅰ / Ⅱ
A (≤ 40)	1813	86.6[a]	9.9	8.7
B (41-50)	2225	86.9[b]	10.6[d]	8.3[f]
C (51-60)	1840	88.6[c]	11.3[e]	7.8[g]
D (≥ 61)	1112	84.0	10.9	7.0[h]

PG Ⅰ: [a]*P* = 0.017, [b]*P* = 0.031, [c]*P* = 0.019 *vs* D group; PG Ⅱ: [d]*P* = 0.018 *vs* A group, [e]*P* = 0.010 *vs* B group; PG Ⅰ / Ⅱ: [f]*P* = 0.003 *vs* A group, [g]*P* = 0.002 *vs* B group, [h]*P* = 0.002 *vs* C group.

age: group A (≤ 40 years of age), group B (41-50 years of age), group C (51-60 years of age) and group D (≥ 61 years of age), respectively. The serum PG levels were compared between different groups (Table 2). The serum PG Ⅱ level increased with age (correlation coefficient: 0.065, *P* = 0.000), while the PG Ⅰ / Ⅱ ratio decreased with age (correlation coefficient: -0.104, *P* = 0.000).

The PG Ⅰ value increased in groups A (86.6 μg/L), B (86.9 μg/L) and C (88.6 μg/L), though the difference was not significant (*P* = 0.754, *P* = 0.683, respectively). The PG Ⅰ value in group D (84.0 μg/L) was significantly lower than that in groups C (*P* = 0.017), B (*P* = 0.031) and A (*P* = 0.019). The PG Ⅱ value in groups A (9.9 μg/L), B (10.6 μg/L) and C (11.3 μg/L) increased significantly in the sequence indicated (*P* = 0.018, *P* = 0.010, respectively). There was no significant difference in the PG Ⅱ level between groups D (10.9 μg/L) and C (*P* = 0.803). The PG Ⅰ / Ⅱ ratio in groups A (8.7), B (8.3), C (7.8) and D (7.0) decreased significantly in the sequence indicated (*P* = 0.003, *P* = 0.002, *P* = 0.002, respectively).

PG levels and kinds of gastric disease

Along the sequence of NOR→SG→GEU→AG→GC groups, the serum PG Ⅰ and PG Ⅱ levels increased and then decreased, while the PG Ⅰ / Ⅱ ratio decreased gradually. The PG Ⅰ and PG Ⅱ levels in the NOR group were lower than those in other groups, while both of them were the highest in GEU. The PG Ⅰ / Ⅱ ratio in the NOR group was higher than that other groups, while it was the lowest in the GC group. Several statistical differences were noticed. The differences in the PG Ⅱ level and the PG Ⅰ / Ⅱ ratio between NOR and other groups were of statistical significance. The differences in the PG Ⅰ / Ⅱ ratio between SG and other groups were of statistical significance. The differences in PG Ⅰ and PG Ⅱ levels in GEU and other groups were of statistical significance. Compared to SG and GEU groups, the differences in PG Ⅱ level and the PG Ⅰ / Ⅱ ratio were of statistical significance in the AG group, while compared to the GC group, the differences in PG, PG Ⅱ level and the PG Ⅰ / Ⅱ ratio had no statistical significance (Tables 3 and 4).

Table 3 Serum PG levels in different gastric disease groups (median)

Gastric disease	Cases (*n*)	PG Ⅰ (μg/L)	PG Ⅱ (μg/L)	PG Ⅰ / Ⅱ
NOR	494	79.3	7.4	10.4
SG	3654	86.0	9.4	9.0
GEU	362	102.5	14.4	7.5
SG-IM	1347	89.9	12.8	6.8
AG	870	84.8	13.0	6.5
GP	73	87.5	13.3	6.1
GD	110	96.9	15.9	5.7
GC	80	84.4	11.3	6.4

Table 4 Results of Mann-Whitney *U* test

Group	*P* value		
	PG Ⅰ	PG Ⅱ	PG Ⅰ / Ⅱ
NOR *vs* SG	0.000	0.000	0.000
NOR *vs* GEU	0.000	0.000	0.000
NOR *vs* AG	0.021	0.000	0.000
NOR *vs* GC	0.892	0.000	0.000
SG *vs* GEU	0.000	0.000	0.000
SG *vs* AG	0.173	0.000	0.000
SG *vs* GC	0.218	0.060	0.000
GEU *vs* AG	0.000	0.017	0.002
GEU *vs* GC	0.000	0.023	0.137
AG *vs* GC	0.422	0.251	0.809

As described in Table 3, atrophic diseases including AG and GC could be taken as the "low group" (L), and non-atrophic diseases including SG and GEU as the "middle group" (M), and NOR as the "high group"(H). The SG-IM could be assigned to the L group. The argument for the categorization of GP and GD was different. Both of them could be assigned to the L group too, since the PG Ⅰ / Ⅱ ratio decreased along with AG→GC→GP→GD, but there was no significant difference between them (*P* = 0.566). Therefore, the median value of the PG Ⅰ / Ⅱ ratio in H (NOR), M (SG + GEU) and L (SG - IM + AG + GC + GP + GD) groups was 10.4, 8.8 and 6.6, respectively, while the corresponding differences between neighboring groups were of statistical significance (*P* = 0.000, *P* = 0.000). More interestingly, it was possible to differentiate three histological groups according to their median PG Ⅰ / Ⅱ ratio: > 10 for normal mucosa, < 7 for atrophic mucosa, and an intermediate value for non-atrophic mucosa.

ROC curves were plotted for each of the serum tests as a predictor of atrophy. The results obtained from the ROC curves could discriminate between patients with atrophy, normal and non-atrophy (Figure 1). The areas under the ROC curves for PG Ⅰ, PG Ⅱ, and the PG Ⅰ / Ⅱ ratio were 0.494, 0.398 and 0.622, respectively, suggesting that the PG Ⅰ / Ⅱ ratio was the only useful biomarker for screening atrophy in this population. The best cut-off point of the PG Ⅰ / Ⅱ ratio for predicting atrophy was 6.9. The corresponding validity parameters were sensitivity (53.2%), specificity (67.5%), PPV (47.3%), and NPV (72.4%). The accuracy of the PG Ⅰ / Ⅱ ratio as a diagnostic test was 62.4%.

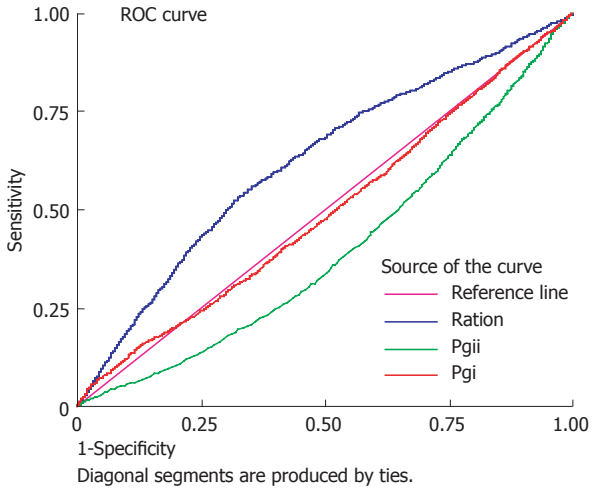

Figure 1 Unadjusted ROC curves for PG Ⅰ, PG Ⅱ and the PG Ⅰ/Ⅱ ratio to discriminate between patients with atrophic lesions and those with normal or inflammatory gastric mucosa.

PG levels and status of H pylori infection

Among the 6990 cases tested, 5285 were positive for *H pylori* infection and 1705 were negative for *H pylori* infection. The PG Ⅰ and PG Ⅱ levels in the positive group were significantly higher than those in the negative group (88.7 μg/L *vs* 81.4 μg/L, $P = 0.000$; 11.4 μg/L *vs* 8.4 μg/L, $P = 0.000$, respectively), while the PG Ⅰ/Ⅱ ratio in the positive group was significantly lower than that in the *H pylori* group (7.7 *vs* 9.6, $P = 0.000$).

Determination of independent variables by multivariable logistic regression

A multivariable logistic regression was performed to determine the dependent variables that could explain the variation of biomarkers. The applied model took four variables of gender, age (< 60 years, ≥ 61 years), gastric mucosal lesion (atrophic or non-atrophic), and *H pylori* infection (+, -) as inputs and considered the PG Ⅰ/Ⅱ ratio (≤ 7, > 7) as the outcome. Results from this regression gave a statistical significance ($\chi^2 = 341.535$, $P = 0.000$). Another qualification for this model was its fitting confidence (13.695, $P = 0.250$). The testing result from this model showed a statistical significance in gender ($P = 0.006$), age ($P = 0.000$), atrophic lesion ($P = 0.000$) and *H pylori* infection ($P = 0.000$). Their corresponding risk factors were male (OR: 1.151, 95% CI: 1.042-1.272), age≥ 61 years (OR: 1.358, 95% CI: 1.188-1.553), atrophic lesion (OR: 2.075, 95% CI: 1.870-2.302) and *H pylori* infection (OR: 1.546, 95% CI: 1.368-1.748) (Table 5). The overall accordance of this model was 64.6%.

Table 5 Multivariable logistic regression of the Pg Ⅰ/Ⅱ ratio

	n	Coeff.	95% CI	*P*-values
Gender				
Female	3535	Baseline		
Male	3455	1.151	1.042-1.272	0.006
Age				
< 60 yr	5878	Baseline		
≥ 61 yr	1112	1.358	1.188-1.553	0.000
Gastric mucosal lesions				
Non-atrophy	4510	Baseline		
Atrophy	2480	2.075	1.870-2.302	0.000
H pylori				
H pylori$^-$	1705	Baseline		
H pylori$^+$	5285	1.546	1.368-1.748	0.000

DISCUSSION

In this study, we reported for the first time the essential serum PG levels in the Chinese population, including some influence influencing factors such as sex, age, the presence of gastric diseases, and *H pylori* infection.

Studies have reported some conflicting results with regard to the correlation between serum PG level and age or sex. For instance, men have higher normal PG Ⅰ values than women[18], which is in agreement with the data on blood donors[19]. In contrast, in 20-70 year old Japanese, serum PG level is dependent on the age and PG Ⅰ increases gradually with age, while the PG Ⅰ/PG Ⅱ ratio decreases significantly[20]. In our study, the PG Ⅰ and PG Ⅱ levels were significantly higher in males than in females. The PG Ⅰ/Ⅱ ratio of the males was significantly lower than that of the females. Both PG Ⅰ and PG Ⅱ levels increased gradually till the age of 60 years, while the PG Ⅰ/Ⅱ ratio showed a significant stage reduction. These findings are in agreement with the reported data[9]. These different age-dependences therefore require a thorough checking of the characteristically different distribution, and difference among populations in application value for serum PG. Another important aspect needing special attention is the identification of its normal distribution in different age and gender groups by stratification.

Serum PG is a well-known indicator and "serological biopsy" of corpus mucosa[21,22]. A cascade of mucosal lesions, from chronic gastritis, atrophy, intestinal metaplasia, to dysplasia has been consistently identified, at least for Lauren's intestinal subtype of GC[23]. In this study, along with the sequence of NOR→SG→GEU →AG→GC, the serum PG Ⅰ and PG Ⅱ levels increased while the PG Ⅰ/Ⅱ ratio decreased. When it came to the atrophic lesion from the non-atrophic lesion, both PG Ⅰ and PG Ⅱ levels had a trend to go down, suggesting that the PG Ⅰ/Ⅱ ratio reflects the development of atrophic lesion on gastric membranes better than either PG Ⅰ or PG Ⅱ alone. The PG Ⅰ/Ⅱ values for atrophic gastritis were significantly lower than those for NOR and non-atrophic lesions, while there was no difference among these atrophic lesions, suggesting that the PG Ⅰ/Ⅱ ratio is an effective parameter for screening individuals at high risk of GC[24-28]. We obtained the best cut-off points of the PG Ⅰ/Ⅱ ratio for detecting GC and its precursors by ROC curve with a sensitivity of 53.2% and a specialty of 67.5%, which can be used for further investigation as a screening tool in the first period.

H pylori infection frequently persists lifelong in the host stomach and is associated with a wide spectrum of human gastric and duodenal diseases, ranging from gastritis to duodenal ulcer, GC and MALT lymphoma[29,30]. In 1994, it was defined as a definite carcinogen by the International

Agency for Research on Cancer[31]. It was reported that *H pylori* stimulates G cells in the antrum, thus increasing the level of gastrin they secreted[32]. Gastrin directly stimulates the main cells and is able to stimulate the synthesis and secretion of PGs, especially PGⅡ by increasing the density of calcium-ion flux, cAMP and phosphoinositide inside the main cells. Serum epidemiological studies showed that *H pylori* infection correlates significantly to the elevated serum PG especially PGⅡ, and the reduced PG Ⅰ/Ⅱ ratio[33]. Our previous study revealed that the incidence of *H pylori* infection is 79.3% in Zhuanghe County[34]. In the present study, the serum PG Ⅰ and Ⅱ levels in positive *H pylori* individuals were significantly higher than those in negative *H pylori* individuals, and the PG Ⅰ/Ⅱ ratio was lower in positive *H pylori* individuals than in negative *H pylori* individuals, confirming that *H pylori* infection influences the serum PG level in residents of Zhuanghe County.

Multivariable logistic regression with the PG Ⅰ/Ⅱ ratio as the outcome showed that the PG Ⅰ/Ⅱ levels were significantly affected if the surveyed samples met any one of the following qualifications: male, age > 60 years, atrophic lesion and *H pylori* infection, showing that all these factors should be taken into account in the screening of GC with serum PGs.

In summary, our results from this population-based study provide the essential characteristics of serum PG in Chinese. The PG Ⅰ/Ⅱ ratio is more suitable for identifying subgroups with different influencing factors, compared with PG Ⅰ or PGⅡ alone. This implies that low PG Ⅰ/Ⅱ ratio can be used as a serological indicator of gastric atrophic diseases.

COMMENTS

Background

Human pepsinogens (PGs) are inactive pro-enzymes for the specific digestive enzyme--pepsin originating from the gastric mucosa, and can be classified biochemically and immunochemically into pepsinogen Ⅰ (PG Ⅰ) and pepsinogen Ⅱ (PG Ⅱ). Serum PG levels are related to the morphologic and functional changes in the stomach, and can be used as a 'serological biopsy'.

Research frontiers

In the present study, we investigated the essential characteristics of serum PG in Chinese, which provides a valuable reference for large-scale surveys of gastric cancer in China.

Innovations and breakthroughs

In this study, we analyzed the essential characteristics of serum PG levels and their influencing factors in residents from the Zhuanghe County in North China. Our results that serum PG skewed from the normal distribution significantly, and was influenced by age, sex, the gastric mucosa lesions and *H pylori* infection. The PG Ⅰ/Ⅱ ratio was more suitable for identifying subgroups with different influencing factors, compared with PG Ⅰ or PGⅡ alone.

Applications

The essential characteristics of serum PG in Chinese are described. The PG Ⅰ/Ⅱ ratio is more suitable for identifying subgroups with different influence factors and can be used as a serological indicator of gastric atrophic diseases.

Peer review

This paper presents the serum pepsinogen values and their influencing factors in a large group of Chinese from North China, which is of certain importance. However, a large room for improvement in its language.

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RAPID COMMUNICATION

Gastrointestinal tract distribution of *Salmonella* enteritidis in orally infected mice with a species-specific fluorescent quantitative polymerase chain reaction

Shu-Xuan Deng, An-Chun Cheng, Ming-Shu Wang, Ping Cao

Shu-Xuan Deng, Ping Cao, Research Center of Poultry Diseases, College of Animal Science and Veterinary Medicine, Sichuan Agricultural University, Yaan 625014, Sichuan Province, China
An-Chun Cheng, Ming-Shu Wang, Research Center of Poultry Diseases, College of Animal Science and Veterinary Medicine, Sichuan Agricultural University; Key Laboratory of Animal Diseases and Human Health of Sichuan Province, Yaan 625014, Sichuan Province, China
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Correspondence to: An-Chun Cheng, Professor, Research Center of Poultry Diseases, College of Animal Science and Veterinary Medicine, Sichuan Agricultural University, Yaan 625014, Sichuan Province, China. chenganchun@vip.163.com
Telephone: +86-835-2885774 Fax: +86-835-2885774
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Abstract

AIM: To identify and understand the regular distribution pattern and primary penetration site for *Salmonella* enteritidis (*S*. enteritidis) in the gastrointestinal tract.

METHODS: Based on the species-specific DNA sequence of *S.* enteritidis from GenBank, a species-specific real-time, fluorescence-based quantitative polymerase chain reaction (FQ-PCR) was developed for the detection of *S.* enteritidis. We used this assay to detect genomic DNA of *S.* enteritidis in the gastrointestinal tract, including duodenum, jejunum, ileum, cecum, colon, rectum, esophagus and stomach, from mice after oral infection.

RESULTS: *S.* enteritidis was consistently detected in all segments of the gastrointestinal tract. The jejunum and ileum were positive at 8 h post inoculation, and the final organ to show a positive result was the stomach at 18 h post inoculation. The copy number of *S.* enteritidis DNA in each tissue reached a peak at 24-36 h post inoculation, with the jejunum, ileum and cecum containing high concentrations of *S.* enteritidis, whereas the duodenum, colon, rectum, stomach and esophagus had low concentrations. *S.* enteritidis began to decrease and vanished at 2 d post inoculation, but it was still present up to 5 d post inoculation in the jejunum, ileum and cecum, without causing apparent symptoms. By 5 d post inoculation, the cecum had significantly higher numbers of *S.* enteritidis than any of the other areas ($P < 0.01$), and this appeared to reflect its function as a repository for *S.* enteritidis.

CONCLUSION: The results provided significant data for clarifying the pathogenic mechanism of *S.* enteritidis in the gastrointestinal tract, and showed that the jejunum, ileum and cecum are the primary sites of invasion in normal mice after oral infection. This study will help to further understanding of the mechanisms of action of *S.* enteritidis.

Key words: Fluorescence-based quantitative polymerase chain reaction; Gastrointestinal tract; *Salmonella* enteritidis

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INTRODUCTION

Salmonella is an enteric pathogen that colonizes the intestinal tract of a variety of animals, especially humans and poultry, and accounts for millions of cases of gastroenteritis and food-borne illness each year[1,2]. *Salmonella* enteritidis (*S*. enteritidis) can be transmitted to humans through the food production chain, and undercooked or raw eggs and poultry meat are a particularly high risk for humans[3]. In the last few decades, *S*. enteritidis has emerged as a major cause of food-borne illness worldwide. As a result of the increased prevalence of *S*. enteritidis and its complex life cycle, identifying the regular distribution pattern of *S*. enteritidis in the gastrointestinal tract will help to understand its mechanism of action.

Previous studies have shown that orally introduced *S*. enteritidis has a rapid transit time through the intestine, and a small proportion of the inoculum establishes itself within the walls of the small intestine and cecum several

days before systemic infection[4,5]. However, the primary site of infection and the route taken by the organism to reach the heart, liver and spleen, are unclear. Infection with *Salmonella* is usually started by oral ingestion of the pathogen, and is followed by bacterial colonization of the gut and invasion of internal tissues. Therefore, knowledge about the mechanisms leading to invasive infections may ultimately lead to new insights into prevention and therapy.

We evaluated the specific fragment (Sdf I) reported by Agron[6], which was screened for using the Supression Subtractive Hybridization method. Sdf I appears to only be found in serovar enteritidis strains, which includes a wide range of clinical and environmental species. Here, based on the specific DNA sequence of serovar enteritidis, a specific fluorescent quantitative polymerase chain reaction (FQ-PCR) for detection of serovar enteritidis was developed and applied to the study of the gastrointestinal tract distribution of *S*. enteritidis.

MATERIALS AND METHODS

Bacterial strains

A total of 14 *Salmonella* strains were included in this study. Most strains were purchased from the National Center for Medical Culture Collection, including *S*. enteritidis (Human, No. 50041), *S*. enteritidis (Human, No. 50040), *S*. enteritidis (Mouse, No. 50338), *Salmonella* Choleraesuis (No. 50191-1), *Salmonella* Typhi (No. 50013), *Salmonella* Typhimurium (No. 50115-13), *Salmonella* Paratyphi (No. 50001-24), *Salmonella* Pullorum (No. 50047-2), *Salmonella* Anatum (No. 50083-4), *Salmonella* Gallinarum (No. 50770), *Salmonella* Dublin (No. 50761). Three strains were isolated and maintained by the Research Center of Poultry Diseases, Collage of Animal Science and Veterinary Medicine of Sichuan Agricultural University, including *S*. enteritidis (Duck, No. MY_1), *S*. enteritidis (Duck, No. SC1) and *S*. enteritidis (Chicken, No. CD1).

Preparation of bacterial samples and generation of standard templates

For the bacterial samples, 5 mL of an overnight culture grown in Luria-Bertani broth was prepared, and then the *S*. enteritidis cells were harvested by centrifugation. The pellet was resuspended in lysozyme solution, followed by lysis using 10% SDS at 60℃ for 1 h. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Then, a 1/10 volume of 3 mol/L sodium acetate and 2 vols absolute ethanol were added, and the nucleic acid was then pelleted by centrifugation, washed with 70% ethanol, and dried under vacuum. The DNA genomic pellet was resuspended in 40 μL TE buffer, and stored at -20℃ until use.

A conventional PCR was carried out using a template from *S*. enteritidis (Mouse, No. 50338), with primers F_1 and R_1 (designed with Sdf I , GenBank Accession No. AF370707.1, generated by TakaRa Biotech, Dalian, China). The primer sequences from 5' to 3' were as follows: F_1, TGTGTTTTATCTGATGCAAGAGG; and R_1, CGTTC TTCTGGTACTTACGATGAC. Amplification was carried out in a total volume of 50 μL, containing 1 μL each primer (25 μmol/L), 1 μL dNTPs (10 mmol/L), 2.5 U Taq DNA Polymerase (TaKaRa Taq; TakaRa Biotech), 5 μL 10 × PCR buffer (with Mg^{2+}, 25 mmol/L) and 2 μL templates, which were then made up to a volume of 50 μL with deionized water. An initial denaturation at 95℃ for 5 min was followed by 32 cycles of denaturation at 94℃ for 30 s, annealing at 52.5℃ for 30 s, and extension at 72℃ for 40 s. Finally, an additional extension was achieved for 10 min at 72℃. The product size was 293 bp.

Finally, the product was gel-excised and quantified with appropriate standards. Its concentration was determined spectrophotometrically using the Bio-Rad Smartspec-3000, according to the manufacturer's instructions. The standards were diluted, divided into aliquots, and frozen before use.

Specific verification of FQ-PCR and its products

The FQ-PCR assay, including volume, Mg^{2+} concentration, probe and primer concentrations, and annealing temperature were optimized. Subsequently, the sensitivity of the assay, the linear range and standard curve were determined by using known amounts of purified template DNA (generated as described above). The primers (F_2 and R_2) and TaqMan-probe (FP) of FQ-PCR were designed using an internal region of the 293 bp sequences (described above, generated by TakaRa Biotech), and were used as follows, from 5' to 3': F_2, TTGATGTGGTTGGTTCGTCACT; R_2, TCCCTGAA TCTGAGAAAGAAAAACTC; and TaqMan-probe (FP), FAM-TGCAGCGAGCATGTTCTGGAAAGC-TAMRA. Amplification was carried out in a total volume of 25 μL, containing 0.6 μL each primer (10 μmol/L), 0.75 μL dNTPs (10 mmol/L), 1.25 U Ex Taq DNA Polymerase (TaKaRa Ex Taq Hot Start Version; TakaRa Biotech), 5 × PCR buffer (free Mg^{2+}) 5 μL, 0.8 μL TaqMan probe (5 μmol/L), 0.5 μL Mg^{2+} (250 mmol/L) and 1 μL templates, then made up to a volume of 25 μL with deionized water. Each run consisted of a 95℃ 5 min hot start, which activated the conjugated polymerase, followed by 45 cycles (50 cycles for sensitivity experiments) with 94℃ denaturation for 30 s, 55℃ annealing for 30 s, and reading the fluorescent signal at this step.

The primers of FQ-PCR (F_2 and R_2) were used for conventional PCR with *S*. enteritidis (Mouse, No. 50338) DNA templates, in order to verify the specific amplification. Amplification was carried out in a total volume of 50 μL, containing 1 μL each primer (25 μmol/L), 1 μL dNTPs (10 mmol/L), 2.5 U Taq DNA polymerase (TaKaRa Taq; TakaRa Biotech), 10 × PCR buffer (with Mg^{2+}, 25 mmol/L) 5 μL, and 2 μL templates, then made up to a volume of 50 μL with deionized water. Initial denaturation at 95℃ for 5 min was followed by 32 cycles of denaturation at 94℃ for 30 s, annealing at 49℃ for 30 s, and extension at 72℃ for 40 s. Finally, an additional extension was achieved for 10 min at 72℃. A 10 μL aliquot of PCR product was electrophoresed on a 1.5% agarose gel for 40-50 min at 80 V, and visualized and photographed under UV illumination.

FQ-PCR standard curve

Based on previous studies, the standard curve was generated as follows[7-9]: Standard DNA from *S*. enteritidis

was used to establish a standard curve. The standard DNA contained amplified target DNA in different quantities which was included in each LightCycler run. The Primers F_2 and R_2 were used for this amplification, and a range of 9×10^8 - 9×10^3 gene copies was used. Concentrations of the standards were measured by fluorometric analysis, and an analysis of key LightCycler measures was performed after each run to verify identical amplification efficiencies and conditions between runs. Finally, these data were used to generate the standard curve through the iCycler IQ Detection System (Bio-Rad, USA) software.

Reproducibility

To evaluate the variability between experiments, four different known concentrations of DNA were amplified by performing the assay described above in triplicate. For each experiment, the crossing point, average crossing point, standard deviation, and coefficient of variation for each assay were calculated.

Sensitivity of FQ-PCR

To determine the detection limit of this FQ-PCR assay, different quantities of standard DNA and different cell numbers of *S*. enteritidis were introduced in the FQ-PCR. A range of 4×10^3 - 4×10^{-1} copies of standard DNA of *S*. enteritidis was added. Then, the results of each concentration were measured by fluorometric analysis. Bacterial cell limit detection was as follows: *S*. enteritidis (Mouse, No. 50338) grown aerobically at 37℃ for 18 h in 5 mL Luria-Bertani broth. Subsequently, 1 mL of overnight culture was harvested, and the pellet was resuspended in 500 μL TE buffer (pH 8.0), from which the number of *S*. enteritidis cells was obtained by conventional viable count method. Then, a 10 fold serial dilution was performed on the overnight culture liquor, and the phenol/chloroform/isoamyl alcohol method was used to extract the DNA template serially from 10^{-6} - 10^{-10} serial dilutions of bacterial liquor. Finally, 1 μL aliquot per concentration of the DNA template was subjected to a test of bacterial cell sensitivity, and the result of the viable count was used to obtain the final result. The FQ-PCR was performed and analyzed as described above.

Specificity of the FQ-PCR

All 14 bacterial strains were used to assess the specificity of the FQ-PCR. The boiling method was used to prepare the DNA template, and 4 μL of this was used in FQ-PCR.

Experimental infection of mice

Our infection model was based on previous studies, which showed that orally introduced *S*. enteritidis had a rapid transit time through the intestine and establishes itself within the walls of the gut for more than 3 d[4,5,10-12,20]. Twenty-eight mice (age 9 wk, specific-pathogen-free) were purchased from the Animal Center of Sichuan University, China. In brief, a group of 14 white mice were orally infected with a virulent S. enteritidis strain (Mouse, No. 50338), at 2.0×10^4 cells per mouse. Another group of 14 white mice was treated with an equal volume of water as a control. Duodenum, jejunum, ileum, cecum, colon, rectum, esophagus and stomach were analyzed by FQ-PCR at different post-inoculation time points, at 30 min, 1, 2, 4, 8, 12, 16, 18, 24 and 36 h, and 2, 3, 4 and 5 d.

One mouse from each group was sacrificed at each time point and its organs were aseptically removed and immediately placed in 1.5 mL labeled snap-cap tubes. The contents were gently removed by lightly squeezing the excised organ and washing twice with 0.85% NaCl. Finally, the tissue samples, without contents in lumens, were placed in 1.5 mL labeled snap-cap tubes and frozen before use.

DNA extraction from the tissue samples was as follows. 0.3 g tissue samples from different segments of the gastrointestinal tract were washed with 0.85% NaCl twice in order to confirm the removal of contents in lumens. Then, the samples were ground in 1.5 mL Eppendorf tubes using a conventional method. The pellet was resuspended in 500 μL TE buffer (pH 8.0) with 10 μL proteinase K (30 mg/mL) and incubated at 37℃ for 3 h. Finally, a conventional phenol/chloroform/isoamyl alcohol method (preparation described above) was used to extract the genomic DNA of *S*. enteritidis from tissue, and 1 μL aliquot of the sample DNA template was used for FQ-PCR detection.

RESULTS

Specific verification of FQ-PCR products

The primers of FQ-PCR were used for conventional PCR with *S*. enteritidis (Mouse, No. 50338) DNA templates, in order to verify the specific amplification. Results showed that the PCR produced an intense band with the expected 130 bp for *S*. enteritidis, which indicated 100% specificity.

FQ-PCR standard curve

One of the main advantages of FQ-PCR is the ability to quantitate unknown samples. With this assay, it is possible to carry out a rapid quantitative analysis of DNA over a wide linear range, with an unknown template. By using a standard template containing from 9×10^8 - 9×10^3 copies, accurate results for a series of samples were obtained, based on the data used to generate the standard curve with the iCycler IQ Detection System. The correlation coefficient for the associated standard curve was 1.000 and PCR efficiency was 97.3%, indicating that the crossing threshold values for the standards fell within an acceptable range. Using the following formula, we could quantitate the number of DNA copies of *S*. enteritidis for unknown samples: $Y = -3.389X + 44.276$ (where Y is the threshold cycle, and X the log of the starting quantity) (Figure 1).

Sensitivity of PCR

A range of 4.0×10^3-4.0×10^{-1} copies of the *S*. enteritidis standard template was used, and the limit of detection was 4 copies/μL. A sensitivity of 6 cfu/mL was obtained when 10-fold serial dilutions of bacterial cell cultures were used as the PCR template (Figure 2).

Reproducibility

Four different, known concentrations of DNA (1.2×10^9-1.2×10^6 copies/μL) were amplified by performing the

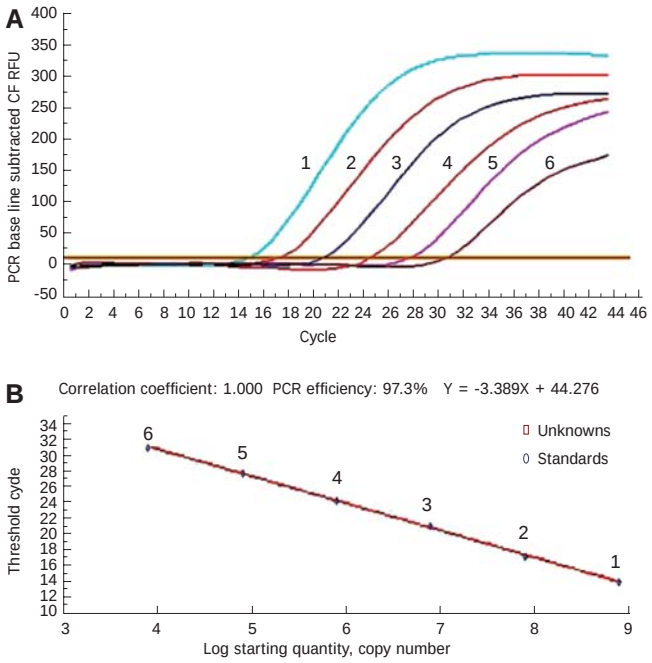

Figure 1 Standard DNA template with 10 fold serial dilutions used to develop the standard curve. 1: 9 ×10^8 copies/μL; 2: 9 × 10^7 copies/μL; 3: 9 × 10^6 copies/μL; 4: 9 × 10^5 copies/μL; 5: 9 × 10^4 copies/μL; 6: 9 × 10^3 copies/μL.

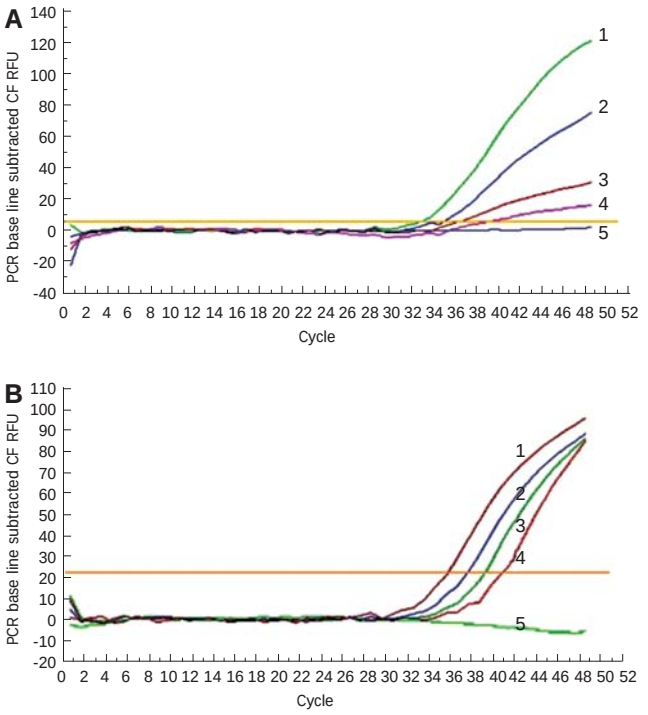

Figure 2 **A**: Sensitivity of FQ-PCR detection using 10 fold serial diluted S. enteritidis (Mouse, 50 338) standard template. 1: 4.0 × 10^3 copies/μL; 2: 4.0 × 10^2 copies/μL; 3: 4.0 × 10^1 copies/μL; 4: 4.0 × 10^0 copies/μL; 5: 4.0 × 10^{-1} copies/μL; **B**: Sensitivity of FQ-PCR detection used 10 fold serial diluted S. Enteritidis (Mouse, 50338) cell number. 1: 6.0 × 10^3 CFU/mL; 2: 6.0 × 10^2 CFU/mL; 3: 6.0 × 10^1 CFU/mL; 4: 6.0 × 10^0 CFU/mL ; 5: 6.0 × 10^{-1} CFU/mL.

assay described above in triplicate. Analysis of these values proved that the assay was reproducible, as the coefficient of variation was statistically low, at < 1.5%. The threshold cycle for each concentration ranged from 1.2 × 10^9-1.2 × 10^6 copies/μL and was different between 0.1-0.3 cycles and highly reproducible (Figure 3).

Specificity of the PCR

All 14 bacterial strains used to assess the specificity of the PCR indicated that only *S. enteritidis* genomic strains showed positive results, while there was no amplification with non-*S.* enteritidis strains (data not shown).

Distribution of S. enteritidis in the gastrointestinal tract

The distribution of *S.* enteritidis within the gastrointestinal tract after oral infection was determined by means of FQ-PCR at intervals on separate segments of gut over a 5-d period. Results showed that the jejunum and ileum were positive at 8 h postinoculation, with approximately 200 copies/g. Then, *S.* enteritidis was consistently detected in all segments of the gastrointestine at 8 h post inoculation, and the last organ to show a positive result was the stomach at 18 h post inoculation. The copy numbers of *S.* enteritidis in each tissue reached a peak at 24-36 h, with the jejunum, ileum and cecum containing high concentrations of *S.* enteritidis, whereas the duodenum, colon, rectum, esophagus and stomach had low concentrations. Numbers of bacteria decreased at 2-3 d, and by 4 d the level of *S.* enteritidis had clearly decreased, with the duodenum, colon, esophagus and stomach not having a positive result. The rectum had about 10 copies/g at 4 d post inoculation, and then the bacteria vanished. The rectum carried the *S.* enteritidis for up to 5 d in others without causing apparent symptoms. Importantly, with respect to the number of

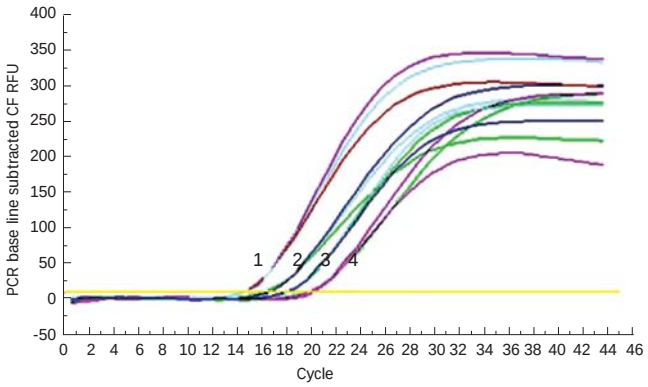

Figure 3 Reproducibility of FQ-PCR. 1: 1.2 × 10^9 copies/μL; 2: 1.2 × 10^8 copies/μL; 3: 1.2 × 10^7 copies/μL ; 4: 1.2 × 10^6 copies/μL.

S. enteritidis cells at 5 d postinoculation, compared to the other organs the cecum had significantly higher numbers of *S.* enteritidis than all of the other regions, with $10^{3.3}$ copies/g, whereas the jejunum had $10^{1.5}$ copies/g, and the ileum had $10^{1.3}$ copies/g ($P < 0.01$). The control group did not have any positive results at any time in any location. The details are given in Table 1.

DISCUSSION

Attachment to host tissues is the first important step for establishing a bacterial infection[13,14]. The fimbria *Sef* 14 is found in a limited number of *Salmonella enterica*

Table 1 Distribution and quantity of *S.* enteritidis in different time and segment of gastrointestinal tract in orally infection mice (lg copies/g)

Time	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum	Esophagus	Stomach
30 min	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1 h	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2 h	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4 h	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8 h	0.0	2.3	2.5	0.0	0.0	0.0	0.0	0.0
12 h	1.2	2.5	2.8	2.1	1.3	1.6	0.0	0.0
16 h	1.5	3.3	3.2	2.6	1.3	1.6	1.1	0.0
18 h	2.2	3.3	3.6	3.0	2.2	1.8	1.2	1.0
24 h	3.0	4.0	4.2	3.9	2.6	2.1	1.4	1.2
36 h	3.3	6.2	6.6	5.4	2.6	3.5	2.6	1.9
2 d	2.2	4.1	4.0	3.9	1.6	2.6	1.6	1.7
3 d	1.2	3.0	2.8	3.6	1.2	1.2	1.3	1.2
4 d	0.0	2.5	2.3	3.4	0.0	1.1	0.0	0.0
5 d	0.0	1.5	1.3	3.3	0.0	0.0	0.0	0.0

serovars, including enteritidis. This surface structure appears to be required for macrophage uptake and survival in intraperitoneal infections[15], in contrast to other *Salmonella* fimbriae that promote binding to host epithelial cells[16]. There is also evidence that quorum sensing plays an important role in the life cycle of *Salmonella* serovar enteritidis[17]. Upon interaction with the intestinal epithelium, *Salmonella* can induce changes in the brush border known as membrane ruffles, survive and multiply there[18]. This study showed that *S.* enteritidis in orally infected mice very quickly reached the intestinal tract, especially the jejunum and ileum. We suggest that penetration by *S.* enteritidis occurs in the upper half of the gastrointestinal tract, which possibly indicates sites that are more easily penetrated by *S.* enteritidis. This finding is compatible with results obtained in previous studies, which showed that *S.* enteritidis has a rapid transit time through the normal mouse intestine; a small proportion of the inoculum establishes itself within the walls of the small intestine, before a systemic infection can be demonstrated[4,5,19]. However, these studies were unable to precisely establish the primary site of bacterial invasion in these animals. From our study, it seems reasonable that the jejunum and ileum are most likely sites for bacterial penetration that is responsible for systemic infection. First, the jejunum and ileum were positive at 8 h post inoculation. Second, the *S.* enteritidis copy number reached a maximum between 8 h and 36 h post inoculation, with copies of *S.* enteritidis being about 10-10 000 times that of other regions. Finally, by 4 d post inoculation, the level of *S.* enteritidis clearly decreased, with no bacteria in the duodenum, colon, esophagus and stomach, but *S.* enteritidis remained in the jejunum, ileum and cecum for up to 5 d post inoculation. At 12 h post inoculation, the *S.* enteritidis in the cecum were increasingly obvious, but less so in the jejunm and ileum (about 10 times fewer) at 24-36 h post inoculation. By 5 d post inoculation, the cecum had significantly higher numbers of *S.* enteritidis than all the other areas, with $10^{3.3}$ copies/g, whereas the jejunum had $10^{1.5}$ copies/g, and the ileum had $10^{1.3}$ copies/g ($P < 0.01$). Therefore, this appeared to reflect the function of the cecum as a repository for *S.* enteritidis and a site for its penetration. By way of contrast, Ozawa[20] has concluded that the primary site of *Salmonella* infection involves the cecum and the large intestine.

Mucosal immunity provides the first line of protection following oral exposure to pathogens. In particular, the involvement of mucosal IgA in protection against salmonellosis has been reported[21]. Secretory IgA limits mucosal colonization by *S.* enteritidis by preventing adherence and subsequent invasion of the bacteria[22,23]. The environment of the gastrointestinal tract can also prevent invasion by *S.* enteritidis to some extent. The lowest concentration of *S.* enteritidis was found in the stomach, and this was the last area in which *S.* enteritidis was detected, which indicates that environmental factors, such as pH, can influence the survival and virulence of *S.* enteritidis. It has been reported that normal host defenses are capable of eliminating > 80% of organisms from the gut within hours[20]. Most of the *Salmonella* in the challenge inoculum had no pathogenic significance, since only a few organisms passed through the mucosa of the ileum during the initial hours of infection. A previous study has shown that *S.* enteritidis colonization in the gastrointestinal tract can persist for as long as 18 wk post inoculation in hens[24]. Nevertheless, these few organisms have the potential to cause a lethal systemic infection[20]. Changes in the microenvironment in the gastrointestinal tract have important implications for understanding the gastrointestinal factors necessary for protection against *S.* enteritidis infection. *S.* enteritidis is distributed evenly along the intestinal tract, and the microenvironment may be a predictor for severity of *S.* enteritidis invasion and infection[25,26].

FQ-PCR has become a potentially powerful alternative in microbiological diagnosis due to its simplicity, rapidity, reproducibility and accuracy. The specific primer-probe combination is a powerful tool for detecting the genetic content of closely related bacterial species[27,28]. A series of sensitivity experiments was performed and proved that the detection limit of this method was 4 copies/μL for standards template, and 6 cfu/mL for bacterial cell number. Four different, known concentrations of DNA were amplified by performing the assay described above in triplicate, and showed a coefficient of variation less than 1.5%. Also, one of the main advantages of FQ-PCR is the ability to quantitate unknown samples. With this assay, it is possible to carry out a rapid quantitative analysis of DNA over a wide linear range, with an unknown template. Simultaneously, variation in results may be due to the extraction efficiency of DNA, the PCR inhibitors, or a large amount of DNA from background organisms.

In conclusion, our results provide significant data for clarifying the pathogenic mechanism of *S.* enteritidis in the gastrointestinal tract, and show that the jejunum, ileum and cecum are the primary sites of invasion in normal mice after oral infection. This study will help to understand the mechanisms of action of *S.* enteritidis.

COMMENTS

Background

There are over 2500 serovars in the genus *Salmonella*. It has been a public health concern for over 100 years, and the incidence of *Salmonella* infections has risen dramatically, especially those caused by *S.* enteritidis. Therefore, knowledge about *Salmonella* infection could be an additional means for decreasing the incidence of infection. Infection with *Salmonella* is usually started by oral ingestion of the pathogen, and is followed by bacterial colonization of the gut and invasion of internal tissues. Therefore, it is necessary to understand its mechanisms of action in the gastrointestinal tract.

Research frontiers

To date, the exact site of primary penetration of *S.* enteritidis in the gut has not been established. FQ-PCR, as a rapid, sensitive technique for precise quantitation of nucleic acid, will be an ideal method to study the distribution of *S.* enteritidis in the gastrointestinal tract.

Innovations and breakthroughs

Previous studies have shown that *S.* enteritidis establishes itself within the walls of the small intestine before a systemic infection can be demonstrated. However, these studies have been unable to identify the regular distribution pattern and the primary site of *S.* enteritidis invasion. In this study, we offered a significant improvement over previous studies, and suggested that the jejunum, ileum and cecum are the primary sites of invasion after oral infection.

Applications

This study will provide significant data for clarifying the pathogenic mechanisms of *S.* enteritidis in the gastrointestinal tract, and may ultimately lead to new insights in prevention and therapy.

Terminology

Standard DNA template: The purified target DNA fragment for FQ-PCR amplification that is usually used to generate standard curves. It can be generated by gel excised directly after a conventional PCR amplification or after the target fragment is cloned using plasmid DNA.

Peer review

This study detected FQ-PCR *S.* enteritidis in different segments of the gastrointestinal tract in mice after oral infection, and demonstrated the regular distribution pattern and primary penetration sites of *S.* enteritidis. The method is simple and accurate, and it may lead to new ways to prevent *S.* enteritidis infection.

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RAPID COMMUNICATION

Effects of a Shuangling Fuzheng anticancer preparation on the proliferation of SGC-7901 cells and immune function in a cyclophosphamide-treated murine model

Hua-Sheng Chen, Jue Chen, De-Li Cui, Yuan-Yuan Zheng, Ai-Hua Xu, Gang Chen, Ling-Chang Jia

Hua-Sheng Chen, Jue Chen, De-Li Cui, Yuan-Yuan Zheng, Ai-Hua Xu, Gang Chen, Yangzhou University Medical College, Yangzhou 225001, Jiangsu Province, China
Ling-Chang Jia, Jiangsu Province Subei People Hospital,Yangzhou 225001, Jiangsu Province, China
Supported by The Society Development Research Programs of Foundation Science and Technology Department of Jiangsu Province, No. BS99382; the Science Basic Research Programs of University of Jiangsu Province, No. 06KJB360131
Correspondence to: Hua-Sheng Chen, Yangzhou University Medical College, Yangzhou 225001, Jiangsu Province, China. yzcwy050130@sina.com
Telephone: +86-514-87884241 Fax: +86-514-87341733
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Abstract

AIM: To study the inhibitory effects of a Shuangling Fuzheng anticancer preparation (SFAP) on the human gastric cancer cell line SGC-7901 *in vitro* as well as its immune-modulated effects in a cyclophosphamide-treated murine model.

METHODS: MTT experiments and immunocytochemistry ABC experiments were performed for detecting the proliferation of SGC-7901 cells *in vitro* and protein expression of c-myc. The staphylococcal protein A (SPA) rosette test was utilized for measuring the ratio of T-lymphocyte subsets from peripheral blood in a cyclophosphamide-treated murine model. Enzyme-linked immunosorbant assay (ELISA) was performed for measuring the levels of serum sIL-2R in treated mice, while immunoturbidimetry was used for measuring the levels of immunoglobulins (Ig).

RESULTS: SFAP (40-640 mg/L, 48 h) inhibited the proliferation of SGC-7901 cells, and a positive correlation was noted between inhibitory effects and dosage. At a dosage of 160-320 mg/L in cultured cells, the expression of c-myc was decreased. SFAP (50-200 mg/kg) increased the percentage of $CD3^+$ and $CD4^+$ T-lymphocytes, the ratio of CD4/CD8, and the contents of Ig such as IgM, IgG or IgA, but decreased the levels of serum sIL-2R in peripheral blood from cyclophosphamide-treated mice.

CONCLUSION: SFAP can inhibit the proliferation of SGC-7901 cells *via* the c-myc gene. In addition, SFAP can modulat the cellular and humoral immunity in cyclophosphamide-induced immunosuppressed mice.

INTRODUCTION

Shuangling Fuzheng anticancer preparation (SFAP) is made through the basic modification of Fuzheng anticancer granules (FAG). Clinical research has shown that FAG have a therapeutic effect for many tumor patients. An empirical study has shown that FAG can prolong the life of tumor-bearing mice and inhibit transplanted tumors. However, this inhibition ratio is lower than 30%[1-3]. Modern pharmacologic studies have shown that atractylodes macrocephalae volatile oil[4], coixenolide[5-7], polysaccharides[8,9] and some other active constituents have anticancer activities. At the precondition of Chinese Medical Syndrome Differentiation, we extracted some of the effective anticancer constituents from FAG and developed SFAP. The main of making SFAP include extraction of volatile oils from atractylodes macrocephalae, coixenolide from coix seed, and polysaccharides from part of Fuzheng traditional Chinese medicines, boiling the other drugs with water as per the traditional method, and concentrating all constituents into a capsule preparation. The results of this experiment showed that the ratio of SFAP anticancer activity was significantly greater than 30%[10]. The present study reports the effects of SFAP on the proliferation of human gastric cancer cells and the expression of related genes *in vitro*, as well as its modulating effects on immune function in a cyclophosphamide-treated murine model.

MATERIALS AND METHODS

Cell line and animals

Human gastric cancer SGC-7901 cells, purchased from the Department of Cellular and Molecular Biology, Shanghai Institute of Biochemistry and Cell Biology, Academia

Sinica, were cultured *in vitro* for 2-3 d. ICR mice (at the age of 6-7 wk, weighing 18-22 g; 50% females) were obtained from Yangzhou University Medical Centre [animal production license number: SCXK (Su) 2002-0009].

Drugs and reagents

SFAP was made from *Smilacis rhizome*, Tuckahoe, honey-fried *Astragalus mongholicus*, *Atractylodes macrocephala*, coix seed, processed *Pinelliae tuber*, *Aurantii nobilis pericarpium* and the like. We extracted volatile oil from *Atractylodes macrocephala*, coixenolide from *Coicis semen*, and polysaccharides from other drugs by boiling in water. They are then mixed at the concentrations specified according to previous formulations. Lastly, the dried drugs were ground into coffee granules. An invention patent for the SFAP preparation protocol has been applied (application No.: 200610161699.5). SFAP was resuspended with RPMI 1640 (GibcoBRL, Maryland, USA) to a suitable dosage, filtrated, sterilized and stored at 4℃ for testing *in vitro*. MTT was from Sigma (St. Louis, MO, USA). Calf serum, trypsin (1:250), ABC immunohistochemical detection kit (concentrated type) and DAB substrate kit were from Huamei Biotechnology Company. Monoclonal mouse anti-human c-*myc* antibodies were provided by Huamei Biotechnology Company. Red blood cell rosette reagent used to restrain the monoclonal antibody hypersusceptibility to CD3, CD4 or CD8 from treated mice was from the Wuhan Biologic Product Institute. The soluble interleukin-2 receptor (sIL-2R) quantitative EIA kit was from Shanghai Senxiong Technology Industry Limited Company. Ig kit was obtained from Shanghai Sun Biological Products Co. Ltd. Injectible cyclophosphamide (CTX, batch number: 20051204) was obtained from Shanxi Jinde Medicine Limited Company. Ultrapure water was self-prepared. Other reagents were all analytical reagents made in China. Adriamycin was from Shenzhen Wanle Pharmaceutical Co. Ltd.

MTT experiment

SGC-7901 cells growing exponentially were digested with 2.5 g/L trypsin, then washed several times with PBS (pH 7.2). They were then counting and RPMI 1640 contained 100 mL/L newborn bovine serum medium in order to adjust cell density to 1×10^8 cells/L. After the final cell suspensions were adjusted the to 100 μL/well, 96-well plates were put into an incubator that containing 50 mL/L CO_2, at 37℃ for 24 h. Then, 100 μL RPMI 1640 containing different concentrations of SFAP (40-640 mg/L) was added to each well. Cells were cultured for 48 h. Four hours before the end of culture, 100 μL supernatant was removed and 10 μL MTT (the final concentration was 5 g/L) was added and cultured for 4 h, to which acid isopropyl alcohol was subsequently added per routine. The absorbance (A) for each well were measured at 570 nm with an ELISA reader. The inhibition ratios were calculated with the following formula: (1 - the mean of treated group/the mean of control group) × 100.

Immunohistochemical detection

Cells were cultured as previously described and the cell density was adjusted to 2×10^8 cells/L. After the above-mentioned cell suspensions were added to a 4 mL culture flask, the flask was placed into an incubator containing 50 mL/L CO_2 at 37℃ for 24 h. Then, culture flasks were randomly divided into groups for different concentrations of SFAP (4 mL), and in the control group RPMI 1640 (4 mL) was added. After continuous culture for 48 h, cells were isolated, digested, and washed. The cells were fixed with 40 g/L formalin, imbedded with paraffin, and then consecutively sliced at a thickness of 3 μm. The paraffin slices were dewaxed and washed with graded alcohol, 3 mL/L H_2O_2 methanol, and incubated for 10 min at room temperature in order to inactivate the endogenous peroxidase activity and eliminate non-specific staining. Next, mouse anti-human c-myc monoclonal antibody attenuated at 1:100 was added, and incubated at 4℃ following the addition of biotinylated sheep anti-mouse IgG. After the above-mentioned mixture was incubated at 37℃ in a moisture-saturated plastic chamber for 30 min, ABC compound was added and incubation was continued for 30 min, followed by three 5-min PBS washes. Staining with DAB was observed by microscope for 5-10 min, after which the cells were washed with tap water for 3 min in order to terminate the reaction. Finally, the slices were stained with haematoxylin, dehydrated, cleared, mounted and observed with a microscope. A light yellow cytolymph indicated positive expression of c-myc protein. Every slice was observed under five high power fields and each positive expression ratio was counted per the following formula: positive expression ratio = (the sum of positive cells/the sum of all the cells) ×100%.

Influence on of SFAPT-lymphocyte subsets from the peripheral blood

The animal study protocol was approved by the Ethics Committee of Yangzhou University Medical College.

A total of 30 ICR mice, half being female, were randomly divided into five groups: NS group, CTX group and three (CTX + SFAP) treatment groups. The ICR mice in the NS group were treated *ig* and *ip* with 0.01 mL/g normal saline (NS) once a day for 5 d; the CTX group was treated ip with 40 mg/kg CTX and ig with NS once a day for 5 d; three (CTX + SFAP) treatment groups were treated ip with CTX 40 mg/kg and *ig* with SFAP, such that the dose was 50, 100 or 200 mg/kg SFAP once a day for 5 d. The Staphylococcal protein A (SPA) rosette test was utilized on the sixth day for measuring the ratio of T-lymphocyte subsets from peripheral blood. The eyes of the mice were removed and venous blood was gathered (about 1 mL), which was then placed into an anticoagulated heparin test tube. One milliliter of NS was added to the test tube. After mixing, 2 mL of T-lymphocyte cell separating medium was added, and then the blood sample was centrifuged for 15 min at 3000 r/min. The intermediate buffy coat was collected and washed three times with Hank's solution. The number of T-lymphocytes was then adjusted to 1000/mm^3. The intermediate lymphocyte suspension (10 μL) was placed into three test tubes and 10 μL of the red blood cell suspension was added to each tube in order to restrain monoclonal antibody hypersusceptibility of CD3, CD4 or CD8 in mice. After incubating the above-mentioned suspension at 37℃ for 30 min, it was centrifuged for

Table 1 Inhibitory effect of SFAP on proliferation of SGC-7901 cells (mean ± SD)

Group	Dose (mg/L)	A value	Rate Inhibition (%)
Control	0	0.290 ± 0.027	0
SFAP	40	0.248 ± 0.055	14.5
SFAP	80	0.239 ± 0.044	17.6
SFAP	160	0.230 ± 0.034	20.7
SFAP	320	0.220 ± 0.041[a]	24.1
SFAP	640	0.212 ± 0.009[b]	26.9
Adriamycin	2	0.125 ± 0.037[b]	56.9

[a]$P < 0.05$, [b]$P < 0.01$ *vs* Control.

Table 2 Influence of SFAP on expression of *c-myc* protein in SGC-7901 cells (mean ± SD)

Group	Dose (mg/L)	Positive c-myc rate (%)
Control	0	22 ± 6
SFAP	160	11 ± 3[b]
SFAP	320	9 ± 3[b]

[b]$P < 0.01$ *vs* control.

3 min at 1000 r/min, made into a slice, dried, and was colored with Gliemsa solution for 30 min. The number of cells positive for CD3, CD4 or CD8 were then observed through a microscope. A T-lymphocytes binding with more than 3 red cells (rosette formation) was defined as positive. More than 200 T-lymphocyte were counted, and the percentage of cells with rosette formation was calculated.

Influence on serum sIL-2R contents

As described above, ICR mice were grouped and treated; in this case, the dose of CTX administered was 10 mg/kg. The eyes were removed, venous blood was gathered (about 1 mL) and blood serum was centrifuged on the sixth day. Per kit instructions, ELISA was used to measure the levels of sIL-2R from blood serum in treated mice. The sample dilution (50 μL) and the solution awaiting detection (50 μL) were put into each well of the reaction plate. After mixing, the reaction plate was stored at 37℃ for 2 h, and was subsequently washed 4-6 times and blotted on filter paper. The first antibody (100 μL) was placed into each well, the reaction plate was stored at 37℃ for 1 h, and then washed as described above. The enzyme-labeled antibody (100 μL) was placed in each well and was stored at 37℃ for 1 h; the reaction plate was then washed as described above. The substrate (100 μL) was placed into each well, and was allowed to react for 5-10 min in the dark at 37℃. Lastly, one drop of stop buffer was put in each well and mixed, from which absorption was then detected at 492 nm. The levels of sIL-2R were determined according to the standard curve of absorption-concentration.

Influence on serum Ig contents

As described above, the dose of CTX was changed to 10 mg/kg. Immunoturbidimetry was utilized for measuring the levels of Ig from blood serum in mice on the sixth day. The eyes were removed, venous blood was collected (1 mL), and the sample was centrifuged. The diluted blood serum (40 μL, 10 μL or 80 μL) was put into a test tube, and 1 mL IgA, IgG or IgM was added to each tube. After mixing, the samples were immersed in a water bath at 37℃ for 15 min, a semi-automatic biochemistry analyzer zero was adjusted to 340 nm with NS, and the absorption for each test was detected. The levels of IgA, IgG or IgM were computed according to the absorption-concentration standard curve.

Table 3 Influence of SFAP on T-lymphocyte subsets from peripheral blood in cyclophosphamide-treated mice

Group	Dose (mg/kg)	CD3 (%)	CD4 (%)	CD8 (%)	CD4/CD8
NS control	0	46.2 ± 4.5	28.5 ± 1.4	17.7 ± 2.16	1.6 ± 0.1
CTX control	40	37.5 ± 2.4[b]	21.5 ± 2.2[b]	16.3 ± 1.6	1.3 ± 0.1[b]
SFAP + CTX	50 + 40	38.7 ± 2.2	22.2 ± 2.3	16.7 ± 2.0	1.3 ± 0.2
SFAP + CTX	100 + 40	39.7 ± 3.1	24.2 ± 2.3	16.2 ± 1.5	1.5 ± 0.1[c]
SFAP + CTX	200 + 40	42.3 ± 2.5[d]	26.7 ± 2.3[d]	16.8 ± 1.5	1.6 ± 0.1[d]

[b]$P < 0.01$ *vs* NS control; [c]$P < 0.05$, [d]$P < 0.01$ *vs* CTX control group.

Statistical analysis

Data are presented as the mean ± SD for all experiments. Comparisons of numerical data were performed using the *t* test, and comparisons of categorical data were performed using χ^2 test.

RESULTS

Effect of SFAP on proliferation of SGC-7901 cells

SFAP (40-640 mg/L, 48 h) inhibited proliferation of SGC-7901 cells in a dose-dependent manner. This effect was statistically significant at dosages of 160 mg/L and 320 mg/L (Table 1).

Effect of SFAP on c-myc protein expression in SGC-7901 cells

SFAP decreased the expression of c-*myc* protein in SGC-7901. This was statistically significant when compared with the control group (Table 2).

Effect of SFAP on distribution of T-lymphocyte subsets from the peripheral blood in mice

CTX (40 mg/kg) decreased the percentage of $CD3^+$ and $CD4^+$ T-lymphocytes and the ratio of CD4/CD8 from the peripheral blood, which was statistically significant compared with the control group. The percentage of the $CD3^+$ and $CD4^+$ T-lymphocytes was increased in all three SFAP dosage groups (50, 100 or 200 mg/kg), with the difference being statisticall significanct in the largest dosage group (200 mg/kg), compared with the CTX group. The moderate and largest dosage groups (100 and 200 mg/kg, respectively) increased the ratio of CD4/CD8 from the peripheral blood, and this was statistically significant compared with the CTX group (Table 3).

Effect of SFAP on serum sIL-2R and Ig contents

CTX (10 mg/kg) increased the levels of sIL-2R in the serum, which was highly significantly different compared with the NS control group ($P < 0.01$). Both moderate and largest dosage

Table 4 Influence of SFAP on serum sIL-2R and Ig levels in cyclophosphamide-treated mice (mean ± SD)

Group	Dose(mg/kg)	sIL-2R (pmol/L)	IgA(g/L)	IgG(g/L)	IgM(g/L)
NS control	0	30.4 ± 2.5	2.92 ± 0.84	8.07 ± 0.94	2.31 ± 0.51
CTX control	10	77.8 ± 8.5[b]	1.77 ± 0.14[b]	6.35 ± 0.63[b]	1.68 ± 0.14[a]
SFAP + CTX	50 + 10	75.9 ± 6.4	2.02 ± 0.31	6.87 ± 0.34	1.82 ± 0.27
SFAP + CTX	100 + 10	59.6 ± 6.1[d]	2.31 ± 0.48[c]	7.70 ± 0.37[d]	2.16 ± 0.33[d]
SFAP + CTX	200 + 10	52.7 ± 5.7[d]	3.11 ± 0.72[d]	7.87 ± 0.59[d]	2.49 ± 0.46[d]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* NS control ; [c]$P < 0.05$, [d]$P < 0.01$ *vs* CTX control.

groups (100 mg/kg and 200 mg/kg) decreased the levels of sIL-2R in serum from mice treated with cyclophosphamide, which was statistically significant compared with the CTX group (Table 4). In addition, CTX (10 mg/kg) inhibited the elaboration of Ig, and was significantly different compared with the NS control group. All three SFAP groups increased the levels of IgA, IgG and IgM in the serum when induced by cyclophosphamide, with statistical significance for the large and moderate dose groups when compared with the CTX control group (Table 4).

DISCUSSION

The target cells in this study were from the cell line SGC-7901. After culturing *in vitro* for 48 h, MTT experiments were utilized to detect SGC-7901 proliferation *in vitro*. The results proved that SFAP (40-640 mg/L) inhibited the proliferation of SGC-7901 and the correlation between the inhibitory effects and the dosage was positive, such that there was a dose-effect relationship. From this observation, we postulate that SFAP directly inhibits the proliferation of SGC-7901 cells. The c-myc protein is the key to controlling the proliferation of these cells, and has been shown to induce tumor cells into multiplication cycles and promote hyperplasia[11-14]. We utilized the immunocytochemistry ABC experiment to detect the influence of SFAP on protein expression of c-myc in SGC-7901 cells. The results proved that SFAP (40-640 mg/L) decreased protein expression of c-myc in these cells. These data imply that c-myc probably affects the regulation of tumor cell proliferation. Considerable evidence has demonstrated that when immunomodulatory mechanisms are suppressed, the incidence rate of the tumor increases and the speed of cancer metastasis and growth quickens. When the malignancy grows constantly or radiotherapy and/or chemotherapy are applied to manage the tumor, the immunomodulatory mechansisms of the patient decline. Thus, there is a close relationship between immunomodulatory activity and the appearance of cancer[15-18].

Cellular immunity is the key to host tumor immunity, and T-lymphocytes are the critical immune component for tumor immunity. The cluster differentiation (CD) antigen is an important immune cell membrane molecule. CD3 antigen is present on the cell membrane of all mature T-lymphocytes in the peripheral blood and is involved in $CD4^+$ and $CD8^+$ T-lymphocyte response. The former is mainly a helper T-lymphocyte, which can also assist B cells to generate antibodies, and the later is chiefly a suppressor T-lymphocyte which can release suppressive cytokines to suppress cellular and humoral immune responses. When T-lymphocyte-mediated cellular immunity is weakened, CD4 is decreased while CD8 is increased, thus the ratio of CD4/CD8 decreases[19-23]. Our experimental results show that SFAP treatment at all used dosages increased the percentage of $CD4^+$ cells in the CTX mouse model, and at the same time increased the ratio of CD4/CD8. IL-2 is an important immune factor secreted by helper T-lymphocytes, which after combining with IL-2R could promote immune cell proliferation and suppress tumor cell division. sIL-2R derives mainly from active T-lymphocyte membrane receptor proteins which could competitively bind to IL-2, thereby restraining the biological activity of IL-2. Thus, sIL-2R is regarded as an important immune suppressor[24-28]. The experimental results in the present study show that SFAP significantly decreased the level of sIL-2R in a CTX mouce model, which suggests that SFAP could enhance cellular immune function in mice inhibited by CTX.

Humoral immunity is critical to host tumor immunity[29]. For example, a variety of antibodies from the blood promote effector cells to recognize and kill tumor cells. Our results show that SFAP is capable of enhancing humoral immune function in mice inhibited by CTX by increasing the serum levels of IgA, IgG and IgM.

Many traditional Chinese medicines derived from the Fuzheng drug are capable of enhancing immune function with few adverse effects; however, the amount of tumor death is small. Traditional cytotoxic drugs are powerful enough to kill tumor cells, but they come with high toxicity, especially with regards to immune and hemotologic function[30]. Thus, many doctors avoid using these kinds of drugs to their patients. The results of our experiments show that SFAP directly inhibits the proliferation of SGC-7901 cells and enhances the cellular and humoral immune function inhibited by CTX. These effects may be beneficial for cancer prevention, anti-cancer therapies, prevention of cancer relapse and metastasis, and avoidance of adverse reactions from radiotherapy and chemotherapy.

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COMMENTS

Background

Malignant tumors are one of the most common causes of mortality in China and the world. While surgical treatment predominates in comprehensive therapy

for tumors, anti-tumor drugs still play an important role. Currently, cytotoxic compounds remain the major constituent of chemotherapy drugs. However, cytotoxic compounds have poor therapeutic effects on the solid tumors and generate higher toxic side-effects and drug resistance. Many Chinese traditional medicines can enhance the immune function of the host, with few toxic side-effects when used in the treatment of tumors.

Research frontiers

Shuangling Fuzheng anticancer preparation (SFAP) consists of Fuzheng and Quxie Chinese traditional medicines. SFAP directly inhibits the proliferation of cancer cells through the action of Fuzheng, and also promotes cellular and humoral immune function in a cyclophosphamide-treatment mouse model through its Quxie action.

Innovations and breakthroughs

Fuzheng anticancer granules (FAG) promote immune function and inhibit transplanted tumors in mice, but the inhibition rate is less than 30%. SFAP has the same constituents of FAG, but improves the action of FAG. At the precondition of Chinese Medical Syndrome Differentiation, the main characteristics of SFAP production were extraction of volatile oils from *Atractylodes macrocephalae*, coixenolide from coix seed, and polysaccharides from part of Fuzheng traditional Chinese medicines, boiling the other drugs with water as per the traditional method, and concentrating all constituents into a capsule preparation. The results of this experiment showed that the ratio of SFAP anticancer activity was significantly greater than 30%. Moreover, SFAP could directly inhibit the proliferation of the cancer cells *in vitro*, and also promote immune function in cyclophosphamide-treated immunosuppressive mice.

Applications

The immune function of the human body has a close association with the development of cancer. On the one hand, when immune function is suppressed, the incidence of cancer increases and the speed of cancer metastasis quickens. On the other hand, when the malignancy grows constantly *in vivo* or radiotherapy or chemotherapy is given to cancer patients, immune function declines as the state of illness worsens. SFAP could directly control tumor cell growth and adjust the balance of cellular and humoral immunity of human body under different conditions. It exerts not only Fuzheng action, but also Quxie action. SFAP may be beneficial for all cancer patients and of applications in cancer prevention, anticancer therapy with few adverse reactions.

Peer review

The Chinese traditional medicine constituents of SFAP are logical and scientific. Its preparation process has many salient features. The preparation exerts not only Fuzheng action, but also Quxie action. The inhibition ratio of SFAP on tumor is higher than common Chinese traditional medicine, and the toxic side-effects are less than those of conventional anti-tumor chemotherapy drugs. As a new compound for anti-tumor therapy, SFAP should be developed as soon as possible.

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RAPID COMMUNICATION

ShRNA-mediated gene silencing of β-catenin inhibits growth of human colon cancer cells

Wen-Sheng Huang, Jian-Ping Wang, Ting Wang, Jie-Yu Fang, Ping Lan, Jin-Ping Ma

Wen-Sheng Huang, Jian-Ping Wang, Ting Wang, Jie-Yu Fang, Ping Lan, Jin-Ping Ma, Surgery Department, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China
Supported by the Guangdong Provincial Scientific Research Grants: No. 2004B30301002, No. 2007B030702012 and No. 04300330
Correspondence to: Jian-Ping Wang, Surgery Department, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, Guangdong Province,
China. chinahwsh2001@yahoo.com.cn
Telephone: +86-20-87755766 Fax: +86-20-87764258
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Abstract

AIM: To observe the gene silencing mediated by the specific shRNA targeted against β-catenin and its effect on cell proliferation and cycle distribution in the human colon cancer cell line Colo205.

METHODS: Two shRNA plasmid vectors against β-catenin were constructed and transfected into Colo205 cells with Lipofectamine™2000. The down-regulations of β-catenin, c-myc and cyclinD1 expressions were detected by RT-PCR and western blot analysis. The cell proliferation inhibitions were determined by MTT assay and soft agar colony formation assay. The effect of these two β-catenin shRNAs on cell cycle distribution and apoptosis was examined by flow cytometry.

RESULTS: These two shRNA vectors targeted against β-catenin efficiently suppressed the expression of β-catenin and its down stream genes, c-myc and cyclinD1. The expression inhibition rates were around 40%-50% either at the mRNA or at the protein level. The shRNA-mediated gene silencing of β-catenin resulted in significant inhibition of cell growth both on the culture plates and in the soft agar. Moreover, the cancer cells showed significant G_0/G_1 arrest and increased apoptosis at 72 h post transfection due to gene silencing.

CONCLUSION: These specific shRNAs targeted against β-catenin could have a gene silencing effect and block the WNT signaling pathway. They could inhibit cell growth, increase apoptosis, and induce cell cycle arrest in Colo205 cells. ShRNA interference against β-catenin is of potential value in gene therapy of colon cancer.

Key words: β-catenin; RNA interference; Apoptosis; Colon cancer; WNT

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INTRODUCTION

Colon cancer is still a serious disease and an abnormal WNT signaling transduction pathway is considered to play an important role during its carcinogenic process. Mutations that activate the WNT pathway have been implicated in colon cancer by turning on genes encoding oncoproteins and cell-cycle regulators. In the absence of the WNT signal, β-catenin is localized in cell-cell junctions, but very low in the cytoplasm and nucleus because excessive β-catenin in cytoplasm is targeted to proteasome-mediated degradation by a complex containing GSK-3β, Axin and APC[1-4]. The binding of WNT proteins to their receptor, frizzled, stabilizes β-catenin by inhibiting the activity of the GSK-3β. Then β-catenin associates with members of the TCF/LEF family and migrates to the nucleus, where this complex activates the transcription of genes, such as c-myc and cyclinD1, which promote proliferation and regulate the cell cycle[5-7]. So, the key step in activating the WNT pathway is the stabilization of β-catenin and its translocation into the nucleus, which may serve as a potential target for colon cancer therapy.

RNA interference (RNAi) is a post-transcriptional gene-silencing mechanism, which was first discovered by Fire and his colleagues in 1998[8]. RNAi is often exploited for specific gene silencing, which is initiated by introduction into cells of double-stranded RNA (dsRNA) that are homologous to the sequence of the target gene. In order to avoid the nonspecific global shutdown of protein synthesis inside the cells and adapt to mammalian cells, the short duplexes of synthetic 21-23 nt RNAs were annealed and then introduced into mammalian cells. This was called small interfering RNA technique (siRNA)[9-11]. In this study, two shRNA plasmid vectors against β-catenin, which could persistently generate siRNA inside cells, were constructed and transfected into the colon cancer cell line Colo205. Its effect on cell proliferation and cycle distribution in this cell line was investigated.

MATERIALS AND METHODS

Cell line and culture

The human colon cancer cell line Colo205, which was kindly provided by my team colleague, Dr Liyong, was cultured in DMEM (Gibco, USA), and supplemented with 10% FBS (Sigma, USA), 100 units/mL penicillin G, and 100 μg/mL streptomycin (Gibco, USA) at 37℃ in a humidified 5% CO_2/95% air atmosphere.

Design of shRNA and plasmid preparation

Plasmid vector Pgenesil was purchased from Wuhan Genesil Biotechnology Co Ltd. Two different targeted sequences were designed to be homologous to the β-catenin mRNA consensus sequence (GeneBank NM_001904). A negative control sequence was also designed as the same process, which had no homology with human beings or mice. The complementary oligonucleotides encoded a hairpin structure with a 21-mer stem and a 9-bp loop. The stem was derived from the mRNA target site. The loop sequence separated the two complementary domains. All the sequences were transcribed with DNA polymerase Ⅲ U6 promoter in plasmid Pgenesil. These two β-catenin plasmids were named Pgenesil-CAT1 and Pgenesil-CAT2 respectively. The negative control plasmid was named Pgenesil-Neg. (Table 1) The transformation of these plasmids into competent cells DH5α and extraction of plasmids followed the routine process.

Plasmid transfection

Approximately, 1×10^6 cells/well were plated in 6-well plates in medium containing 10% FBS to grow overnight to 80%-90% confluency. Transfection of the shRNA oligonucleotides was performed by using LipofectamineTM2000 (Invitrogen, USA). Colo205 cells were divided into blank control group, negative control group and two test groups (CAT1 and CAT2). Only LipofectamineTM2000 was used for transfection in the blank control group. Plasmid Pgenesil-Neg was used for transfection in the negative control group. Plasmids Pgenesil-CAT1 and Pgenesil-CAT2 were used for transfection in the test groups (CAT1 and CAT2) respectively. In the above three groups, the cells were all transfected with the mixture of plasmid and Lipofectamine TM2000 (1:3) in 2 mL serum-free medium. At 6 h post-transfection, 500 μL FBS/well was added. At 26 h after transfection, the medium was replaced by normal medium containing 10% FBS and antibiotics up to 72 h post-transfection.

RNA isolation and semiquantitative reverse transcription PCR(RT-PCR)

At forty-eight hours post-transfection, 1×10^6 cells were collected and total RNA was extracted, using the Trizol reagent (Invitrogen, USA) under its operational instruction. The concentration and purity of the total RNA were detected with UV spectrophotometer analysis at 260 nm and the electrophoresis detection showed good quality of purified RNA. RT-PCR was performed by the two-step method. Synthesis of cDNA was performed by using the cDNA synthesis kit (Fermentas, USA) and following the manufacturer's instructions. For quantitative analysis of β-catenin, c-myc and cyclinD1 mRNA, the expression of house keeping gene, β-actin mRNA, was used as an internal standard. Primer pairs were designed according to the sequences in the GenBank (Table 2).

Table 1 Oligonucleotides sequences of β-catenin specific and negative control siRNA

Name	Sequence of siRNA	Target sites
Pgenesil-CAT1	AACAGTCTTACCTGGACTCTG	0290-0310
Pgenesil-CAT2	AAAGGCAATCCTGAGGAAGAG	0355-0375
Pgenesil-Neg	GACTTCATAAGGCGCATGC	No homology

The PCR fragments were separated and visualized in 1.5% agarose gels stained with ethidium bromide. Semiquantitative analysis was performed with the Bio-1D gel analysis software. All experiments were done in triplicate. The ratio of the photodensity of the RT-PCR product of goal gene and β-actin was used to identify the expression intensity of goal gene. The inhibition rate of goal gene expression was calculated with the following formula: inhibition rate of goal gene expression (IR) = $(1 - E_1/E_2) \times 100\%$. E_1: the expression intensity of goal gene in the observation group; E_2: the expression intensity of goal gene in the blank control group.

Western blot

The cells were divided and transfected as above for 48 h. After collection from the culture medium, the cells were washed three times with PBS and lysed in 150 μL of ice-cold Tris buffer (50 mmol/L, pH 7.5). The Tris buffer contains 5 mmol/L edetic acid, 150 mmol/L NaCl, 0.1% NP-40, 0.1% SDS, 2.0 g/L aprotinin, 0.02% NaN_3, 0.2 mmol/L PMSF, and antiprotease mixture. Then the cells were sonicated, and centrifuged at 12000 × *g* for 15 min. The Coomassie brilliant blue G-250 was used to determine the concentration of protein in each lysate. Loading buffer was added to each lysate, which was subsequently boiled for 3 min and then electrophoresed by SDS-PAGE. The proteins were mixed with 2 × loading buffer with the same volume before electrophoresis. After transferring onto nitrocellulose, proteins were incubated with antibodies (β-catenin, c-myc and cyclinD1, purchased from Santa Cruz, USA) and then with peroxidase-conjugated secondary antibody (Bolster, China). Detection was performed with an enhanced chemiluminescent agent. Signals were detected with the ECL Test Kit (Maixin, China). The β-actin staining served as the internal standard for all membranes. The inhibition rate calculating formula is the same as that described in the RT-PCR section. All experiments were done in triplicate.

MTT assay

The transfected cells were seeded in 96-well plates (1×10^4/mL) and allowed to attach for about 24 h. The MTT (Sigma, USA) was dissolved in PBS at a concentration of 5 mg/mL and filtered. Ten microlitres of stock solution were added to 100 microlitres of medium in each well. Then, the plates were incubated for 4 h at 37℃. After loading of MTT, the medium was replaced with 100 μL DMSO and left for 30 min at room temperature for colour development. The 96-well plates were read by an

Table 2 Oligonucleotides sequences of primer pairs

Goal gene	Upstream primer	Downstream primer	PCR frag(bp)
β-actin	5'-TCCTGTGGATCCACGAAACT-3'	5'-GAAGCATTTGCGGTGGACGAT-3'	330
β-catenin	5'-AGATGCAGCAACTAAACAGGA-3'	5'-GTACTACATTTTAAGCCATCT-3'	290
C-myc	5'-TACCCTCTCAACGACAGCAG-3'	5'-TCTTGACA TTCTCCTCGGTG-3'	477
CyclinD1	5'-GCCAACCTCCTCAACGACCGG-3'	5'-GTCCATGTTCTGCTGGGCCTG-3'	744

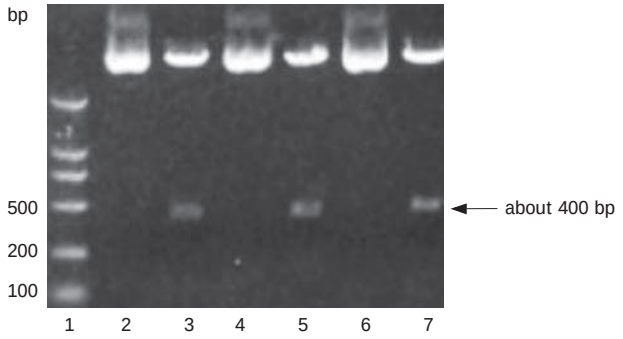

Figure 1 Identification of plasmid Pgenesil by restriction enzyme digestion. Lane 1: Marker; Lane 2: Plasmid Pgenesil-CAT1; Lane 3: Plasmid Pgenesil-CAT1 digested by *SalI*; Lane 4: Plasmid Pgenesil-CAT2; Lane 5: plasmid Pgenesil-CAT2 digested by *SalI*; Lane 6: Plasmid Pgenesil-Negative; Lane 7: Plasmid Pgenesil-Negative digested by *SalI*.

enzyme-linked immunosorbent assay reader (570 nm, DG-3022A, USA) to determine absorbance values (A). The absorbance values were determined at 36, 48, 60, 72 h after transfection in each group respectively. The experiments were repeated three times. The Inhibition rate was calculated by the following formula. Inhibition rate (IR) = [1 - A_1/A_2] × 100% A_1: absorbance value of the observation group A_2: absorbance value of the control group.

Soft agar colony formation assays

At 24 h after transfection as above, the cells of different groups were mixed with the above mentioned culture media containing 0.4% agar. Then 1000 cells were resuspended and seeded in triplicate into 6-well plates coated with 0.5% agar in DMEM containing 10% FBS. After 10 d incubation, the colonies were counted. Colony efficiency (CE) was determined by counting the number of colonies greater than 60 μm in diameter at × 100 magnification in each plate.

Flow cytometry

Cells of each group seeded in culture plates were transfected as described above. After incubation for 72 h, cells were harvested by trypsinization and centrifugation, washed with cold PBS and fixed overnight with 80% ethanol at -20℃. The fixed cells were collected, washed and resuspended in PBS containing 10 μg/mL propidium iodide (Sigma, USA) and 100 μg/mL RNase A (Takara, Dalian, China), then incubated at 4℃ for at least 30 min avoiding light to eliminate the intracellular RNA. Subsequent analyses were performed by flow cytometry (Becton Dickinson, USA). The experiments were repeated three times.

Table 3 The mRNA expression intensities of genes in different groups normalized by β-actin

Goal gene	Blank Control	Negative	CAT1	CAT2
β-catenin	0.98 ± 0.02	0.96 ± 0.03	0.51 ± 0.03	0.55 ± 0.01
C-myc	0.97 ± 0.01	0.96 ± 0.02	0.48 ± 0.04	0.52 ± 0.01
CyclinD1	0.98 ± 0.02	0.97 ± 0.03	0.47 ± 0.02	0.50 ± 0.02
P		[a]*P* > 0.05	[a]*P* > 0.05	[a]*P* > 0.05

[a]*P*: Observation group *vs* blank control group.

Statistical analysis

The results were expressed as mean ± SD. The data were treated by one-way analysis of variance and Student's *t*-test to determine statistical significance with SPSS 11.5 statistic software. $P < 0.05$ was considered statistically significant.

RESULTS

Construction and verification of plasmids

The multiclone sites of plasmid Pgenesil were as follows: *HindIII-ShRNA-BamHI-U6Promotor-EcoRI-SalI-XbaI-DraIII*. *A SalI* site for plasmid Pgenesil was designed in the inserted fragments between the sites of *BamHI* and *HindIII*. If the insertion was correct, a band about 400 bp should be cut off by *SalI*. The results of digestion with restriction endonucleases and sequencing showed correct plasmids (Figure 1).

Inhibition of mRNA expression by β-catenin shRNA

The mRNA expression intensities of goal genes, inhibited by specific shRNAs in the colon cancer cells, were analyzed by semiquantitive RT-PCR. The mRNA levels were normalized by internal control β-actin (Figure 2). At forty-eight hours post-transfection, the expression intensities of β-catenin mRNAs in the blank control, negative control and test groups (CAT1, CAT2) were 0.98 ± 0.02, 0.96 ± 0.03, 0.51 ± 0.03 and 0.55 ± 0.01, respectively. The other genes' mRNA expression intensities were shown in the table (Table 3). The statistical analysis showed that β-catenin mRNAs of Colo205 cells in the CAT1 and CAT2 groups were down regulated significantly after transfection with either plasmids Pgenesil-CAT1 or Pgenesil-CAT2, compared with that in the blank group ($P < 0.05$). The inhibition rates were 47.89% and 43.91% in the CAT1 and CAT2 group, respectively (Figure 2). But there is no significant difference between these two groups. The plasmid Pgenesil-Neg had no significant inhibitive effect on the expression of β-catenin mRNA ($P > 0.05$, *vs* blank) (Table 3). The statistical results of gene c-myc and cyclinD1 were similar to that of β-catenin. (Table 3).

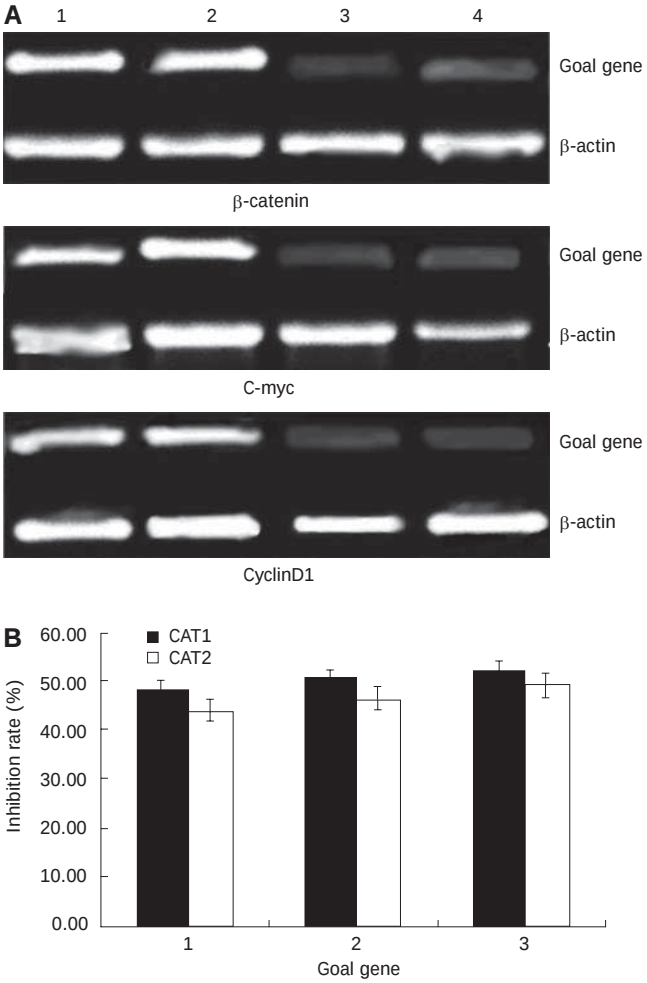

Figure 2 RNA interference-mediated suppression of cellular mRNA. **A**: The mRNA expressions of different goal genes in colon cancer cells at 48 h post-transfection were examined by semiquantitive RT-PCR. The β-actin gene served as internal control. Lane 1: Blank control; Lane 2: Negative control; Lane 3: Pgenesil-CAT; Lane 4: Pgenesil-CAT2; **B**: The inhibition rates of different genes down-regulated by plasmid Pgenesil-CAT1 and Pgenesil-CAT2. Goal gene 1: β-catenin; 2: c-myc; 3: cyclinD1.

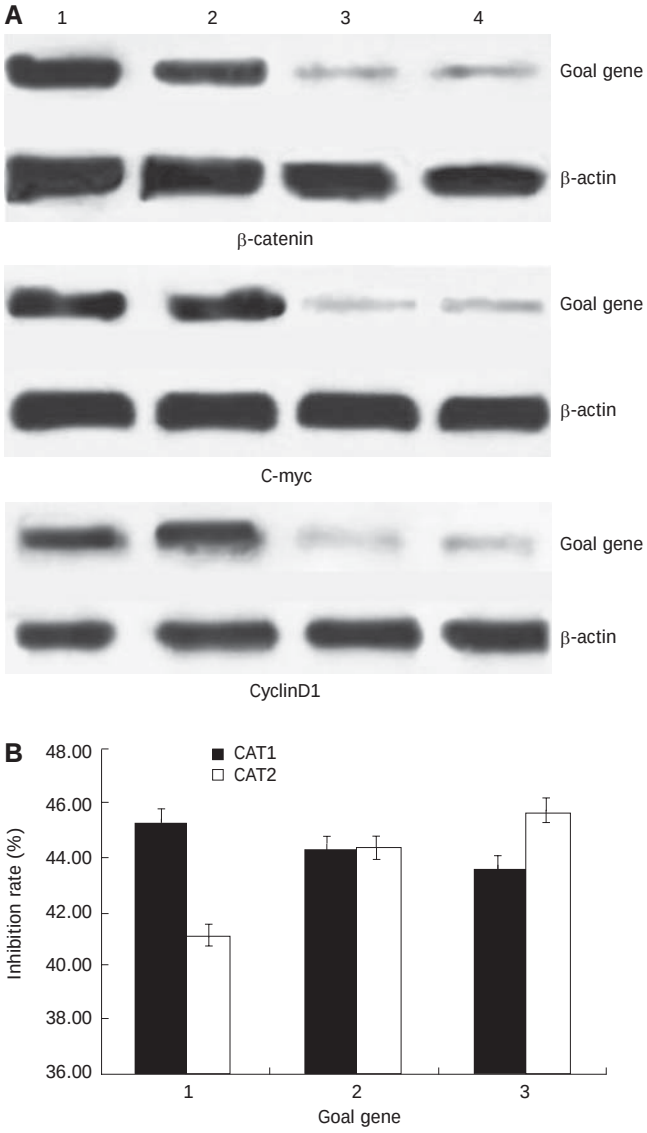

Figure 3 RNA interference-mediated suppression of cellular protein. **A**: Western blot analysis for different goal genes. The β-actin gene served as internal control. Lane 1: Blank control; Lane 2: Negative control; Lane 3: Pgenesil-CAT1; Lane 4: Pgenesil-CAT2 group; **B**: The inhibition rates of different genes down-regulated by plasmids Pgenesil-CAT1 and Pgenesil-CAT2. Goal gene 1: β-catenin; 2: c-myc; 3: cyclinD1.

Inhibition of protein expression by β-catenin shRNA

The down-regulated efficiencies of protein expressions inhibited by specific shRNAs in Colo205 cells were analyzed by western blot. The protein levels were normalized by internal control β-actin. (Figure 3) At forty-eight hours post-transfection, all the protein expression ratios (protein *vs* β-actin) were calculated and shown in Figure 3 and Table 4. For example, the ratio of β-catenin /β-actin were 0.95 ± 0.02, 0.89 ± 0.04, 0.52 ± 0.02 and 0.56 ± 0.03, in the blank control, negative control and test groups (CAT1,CAT2) respectively. The statistical analysis showed that both the plasmid Pgenesil-CAT1 and Pgenesil-CAT2 could have a significant down-regulation effect on the protein expression of β-catenin in Colo205 cells ($P < 0.05$, *vs* blank). But there is no significant difference between these two groups. The similar statistical results could be detected on the protein expression of gene c-myc and cyclinD1 ($P < 0.05$, *vs* blank). The inhibition rates were around 40%-50%. Reasonably, the plasmid Pgenesil-Neg had no inhibitive effect on any gene of β-catenin, c-myc and cyclinD1 ($P > 0.05$, *vs* blank).

Inhibition of cell proliferation by β-catenin shRNA

The proliferation of Colo205 cells inhibited by specific shRNAs was analyzed by the MTT assay. The results were shown in Table 5. The statistical analysis showed that both the plasmids Pgenesil-CAT1 and Pgenesil-CAT2 could have significant inhibitive effects on the proliferation of Colo205 cells, compared with the blank group at 72 h after transfection ($P < 0.05$, *vs* Blank). But the plasmids Pgenesil-Neg had no significant inhibitive effect on cell proliferation ($P > 0.05$, *vs* Blank).

Inhibition of anchorage-independent proliferation of Colo205 cells by β-catenin shRNA

The anchorage-independent proliferation of Colo205 cells inhibited by specific shRNAs was analyzed by soft agar colony formation assays. The average numbers of colonies in the blank, negative, CAT1, CAT2 group were

Table 4 The western blot analysis for genes in different groups normalized by β-actin

Goal gene	Blank Control	Negative	CAT1	CAT2
β-catenin	0.95 ± 0.02	0.89 ± 0.04	0.52 ± 0.02	0.56 ± 0.03
C-myc	0.97 ± 0.01	0.92 ± 0.02	0.54 ± 0.01	0.54 ± 0.04
CyclinD1	0.94 ± 0.03	0.95 ± 0.01	0.53 ± 0.02	0.51 ± 0.01
P		[a]*P* > 0.05	[a]*P* < 0.05	[a]*P* < 0.05

[a]*P*: Observation group *vs* blank control group.

Table 5 The Inhibition rate of cell proliferation in different group

	Blank Control (%)	Negative (%)	CAT1 (%)	CAT2 (%)
36 h	10	14	21	18
48 h	13	17	28	24
60 h	15	19	39	33
72 h	18	21[c]	54[a]	47[a]

[a]*P* < 0.05 (*vs* Blank), [c]*P* > 0.05 (*vs* Blank).

46, 43, 9 and 11 respectively. The Colo205 cells in the CAT1 and CAT2 groups formed significantly less colonies (4-5 fold decrease) in soft agar than the cells in the blank and negative control groups did ($P < 0.05$, *vs* Blank). At the same time we observed that most of the colonies in the CAT1 and CAT2 groups were much smaller than those in the blank and negative control groups. All these results suggested that reductions in β-catenin protein level induced by the β-catenin gene silencing decreased the abilities of Colo205 cells to form colonies in soft agar.

Effect of β-catenin shRNA on cell cycle distribution and apoptosis

In comparison to the blank control group, the CAT1 and CAT2 groups both showed cell accumulation in the G_0/G_1 phase and reduction in the phases of S and G_2/M. Moreover, the cells with sub- G_1 DNA content also increased and apoptosis rates were around 5% in these two groups. No statistically significant difference was found between the negative control group and the blank control group (Table 6).

Table 6 Effect of shRNA interference on cell cycle distribution and apoptosis

Group	G_0/G_1 (%)	S (%)	G_2/M (%)	Apoptosis (%)
Blank	36.51 ± 2.52	47.32 ± 3.31	16.45 ± 1.52	0.78 ± 0.11
Negative	40.32 ± 2.74[c]	43.21 ± 2.12	16.57 ± 1.83	1.05 ± 0.15[c]
CAT1	81.71 ± 5.73[a]	14.34 ± 1.34	4.55 ± 0.35	5.21 ± 0.47[a]
CAT2	78.33 ± 5.32[a]	17.51 ± 1.81	5.31 ± 0.57	4.89 ± 0.38[a]

[a]*P* < 0.05 (*vs* Blank); [c]*P* > 0.05 (*vs* Blank).

DISCUSSION

RNA interference is a ubiquitous mechanism of eukaryotic gene regulation and an excellent strategy for specific gene silencing. The specificity of RNAi is determined by 21-23 nt RNA duplexes, referred to as micro-RNA (miRNA) or small interfering RNAs (siRNA)[12]. ShRNA is formed by hairpin structures and stretches of double-stranded RNA, which will be cleaved by the ribonuclease dicer to produce mature miRNA inside the targeted cells. After unwinding, one of the strands becomes incorporated into the RNA-induced silencing complex (RISC) and guides the destruction or repression of complementary mRNA[13]. Recently the vector-based approach of shRNA interference has been developed in order to achieve stable, long-term, and highly specific suppression of gene expression in mammalian cells[14]. These shRNA expression vectors have many advantages: they can be stably introduced into cells and persistently effective, either as selectable plasmids or as retroviruses. They are relatively cheap to generate[15,16]. These vectors are often under the control of an RNA polymerase Ⅲ promoter such as U6 or H1[17]. They can transcribe and generate siRNA continuously and the gene silencing effect can last persistently inside the cells. These findings have opened a broad new avenue for the analysis of gene function and gene therapy[18].

The coordination of cell proliferation and apoptosis in cells is one of the hot spots in cancer research. The balance between cell proliferation and apoptosis, and the distribution of cell cycles play very important roles in the carcinogenesis process of colon cancer. The WNT/β-catenin signaling transduction pathway is a highly conservative pathway in the development of colon cancer, which regulates intestinal epithelial proliferation and patterning[19,20]. Mutations that activate the WNT signaling pathway cause the hyper-proliferation of intestinal crypt progenitors and a series of consequential changes finally lead to the occurrence of colon cancer[21,22]. β-catenin is the key component in this pathway. The increased levels of β-catenin frequently found in both premalignant and malignant cells are associated with increased rates of cellular proliferation[23]. Moreover, modest over-expression of β-catenin in epithelial cells can lead to increased proliferation and result in transformation[24-26]. It has been reported that the excessive expression of stabilized β-catenin leads to tumorigenesis in the colorectum, central nervous system, skin and other tissues[27-30]. So, β-catenin has attracted much attention as gene therapy target for carcinomas, including colon cancer. β-catenin has been successfully down-regulated by RNAi in some previous study. For example, Udit N Verma *et al* synthesized siRNA and interfere with the β-catenin gene in the cell line SW480 and HCT116[31]. However, the down-regulation of β-catenin expression by RNAi in the colon cancer Colo205 cell line has not been reported before. Furthermore their research used synthesized small interference RNA as a gene silencing tool, which just transiently suppressed the gene expression and was often limited to cells that are easily transfected[17]. In our study, we did differently by using the vector-based RNA interference technique. According to the design of the shRNA, we selected two shRNA sequences from the β-catenin (CTNNB1) gene and used the plasmid Pgenesil as the vector. The plasmid Pgenesil vector contains the EGFP gene that makes it convenient to observe the result of transfection.

When successfully transfected into the colon cancer cells, the vector Pgenesil could transcribe and generate the interfering RNAs continually under the control of the U6 promotor. So the concentration of intracellular interfering RNA oligonucleotides can remain stable long after transfection. That is what we thought to be the mechanism for the persistently inhibitive effect of the vector-based interference technique. This is the major advantage against the common siRNA technique.

In this study, these two β-catenin specific shRNAs showed their evident effect on silencing the β-catenin gene. Our results demonstrated that after transfection with the β-catenin specific shRNA vectors, β-catenin mRNA and protein expression were significantly suppressed, detected by RT-PCR and western blot. At the same time, we could observe that the genes c-myc and cyclinD were also significantly depressed both at the mRNA and protein levels due to the β-catenin gene silencing. This result confirmed that the c-myc and cyclinD1 genes are the downstream genes regulated by β-catenin. Gene silencing of β-catenin can sequentially lead to the silence of c-myc and cyclinD. Furthermore, no significant differences of β-catenin mRNA or protein expression were observed between these two vectors (CAT1 and CAT2). On the other hand, the negative control plasmid had no inhibitory effect on β-catenin mRNA and protein expression. Neither did it in the genes c-myc and cyclinD1. These results together confirmed that the inhibitions of β-catenin and its downstream genes were specifically induced by these shRNAs aimed at β-catenin. The MTT assays demonstrated that the proliferation curves of the cells were similar in the blank and negative control groups. Viability of cells transfected with the negative control vectors did not significantly decline compared with that of the blank control group. Thus the vector plasmid Pgenesil could be used as a safe and non-toxic carrier for shRNA interference. However, cell proliferation in test groups, which were transfected with β-catenin specific shRNAs, showed increasingly slow down in relation to the time after transfection. At 72 h post-transfection, the inhibitive rates of cell proliferation were 54% and 47% in the CAT1 and CAT2 group respectively, which have significant differences compared with that of the blank control group. As for the anchorage-independent proliferation, we could also detect the significant reduction of colonies in either amounts or sizes in soft agar in the test groups. The MTT and soft agar assay together revealed that these two β-catenin specific shRNAs possessed the anti-proliferation function. Our flow cytometry results indicated that the cell growth inhibition by β-catenin shRNA was due to the cell cycle G_0/G_1 arrest and induction of apoptosis. This was somewhat controversial to Udit N Verma's report, which implied that the decreased cell proliferation did not depend on the mechanism of increased apoptosis[31]. We think that the controversy may be due to different cell lines. In our opinion, we think that the imbalance between cell proliferation and apoptosis induced by the β-catenin shRNA could be possibly explained by mechanisms of β-catenin gene silencing and its consequential downstream genes silencing, such as c-myc and cyclinD1, which regulate the cell cycle transition checkpoints and cellular proliferations. As we know, c-myc is a positive regulator of G1-specific cyclin-dependent kinases (CDKs). Even in quiescent cells, c-myc activation is sufficient to induce cell cycle entry in the absence of growth factors[32,33]. CyclinD1 is a regulatory kinase that is a critical modulator of progression through the G1 to S phase transition of the cell cycle. When quiescent cells re-enter the cell cycle and divide, cyclinD1 is the first cyclin to be activated[34,35]. Overexpression of these two genes may stimulate the cells to overcome the cell cycle checkpoints and enhance cell proliferation[36]. Inhibitions of these two genes may result in cell cycle arrest and increased apoptosis, thus inhibiting cell growth. But whether this mechanism is of universality in other colon cancer cell lines or even malignant tumors, further detailed investigations need to be carried out.

In conclusion, our experiments showed that the shRNA targeted against β-catenin could specifically mediate the β-catenin gene silencing and consequentially suppress the expression of its downstream genes, c-myc and cyclinD1. These genes' silencing effect could efficiently inhibit the growth of colon cancer Colo205 cells. The shRNA interference targeted against β-catenin may have potential therapeutic utility in human colon cancer.

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COMMENTS

Background

Abnormal WNT signaling transduction pathway is considered to play an important role during the carcinogenesis process. The key step in activating WNT pathway is the stabilization of β-catenin and its translocation into nucleus, which may serve as a potential target for colon cancer therapy. RNAi is often exploited for specific gene silencing, which provides a strategy for cancer gene therapy. Therefore, we would do some basic research for colon cancer therapy by using this technique.

Research frontiers

The coordination of cell proliferation and apoptosis in cells is one of the hotspots in cancer research. The balance between cell proliferation and apoptosis, and the distribution of cell cycles play very important roles in the carcinogenesis process of colon cancer. β-catenin is the key component in this process. Thus β-catenin has attracted much attention as gene therapy target for colon cancer.

Innovations and breakthrough

In this study, two β-catenin specific shRNAs showed their evident effect on silencing the β-catenin gene. The results also indicated that the cell growth inhibition by β-catenin shRNA was due to the cell cycle G_0/G_1 arrest and induction of apoptosis, although this was somewhat different from other's report.

Applications

The shRNA interference targeted against β-catenin may have potential therapeutic utility in human colon cancer.

Terminology

ShRNA: ShRNA is the short form of "short hairpin RNA". It is formed by hairpin structures and stretches of double-stranded RNA, which will be cleaved by the ribonuclease dicer to produce mature miRNA inside the targeted cells.

Peer review

This paper investigated the gene silencing mediated by the specific shRNA

targeted against β-catenin and its effect on cell proliferation and cycle distribution in human colon cancer cell line Colo205. The authors conclude that The specific shRNAs targeted against β-catenin could have gene silencing effect and block the WNT signaling pathway. They could inhibit cell growth, increase apoptosis, and induce cell cycle arrest in Colo205 cells. ShRNA interference against β-catenin is of potential value in gene therapy of colon cancer.

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RAPID COMMUNICATION

Predictive value of D-dimer for portal vein thrombosis after portal hypertension surgery in hepatitis B virus-related cirrhosis

Mei-Hai Deng, Bo Liu, He-Ping Fang, Wei-Dong Pan, Zhao-Feng Tang, Peng Deng, Yue-Si Zhong, Rui-Yun Xu

Mei-Hai Deng, Bo Liu, He-Ping Fang, Wei-Dong Pan, Zhao-Feng Tang, Peng Deng, Yue-Si Zhong, Rui-Yun Xu, Department of Hepato-biliary Surgery, Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510630, Guangdong Province, China
Supported by Technology Support Fund of Guangdong Province, No. 2004B35001007
Correspondence to: Dr. Mei-Hai Deng, Department of Hepato-biliary Surgery, Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510630, Guangdong Province, China. drdmh@medmail.com.cn
Telephone: +86-20-85613318 Fax: +86-20-85262937
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Abstract

AIM: To evaluate the predictive value of D-dimer as a predictive indicator of portal vein thrombosis (PVT) after portal hypertension surgery in hepatitis B virus-related cirrhosis.

METHODS: A prospective study was carried out in 52 patients who had undergone surgery for portal hypertension in hepatitis B virus-related cirrhosis. Changes in perioperative dynamic D-dimer were observed. The sensitivity, specificity, positive predictive values and negative predictive values of D-dimer were calculated, and ROC curves were analyzed.

RESULTS: The D-dimer levels in the group developing postoperative PVT was significantly higher than those in the group not developing PVT (P = 0.001), and the ROC semi-quantitative and qualitative analysis of D-dimer showed a moderate predictive value in PVT (semi-quantitative value Az = 0.794, P = 0.000; qualitative analysis: Az = 0.739, P = 0.001).

CONCLUSION: Dynamic monitoring of D-dimer levels in patients with portal hypertension after surgery can help early diagnosis of PVT, as in cases where the D-dimer levels steadily increase and exceed 16 μg/mL, the possibility of PVT is very high.

Key words: Portal hypertension; Portal vein thrombosis; Splenectomy; D-dimer; Diagnosis

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INTRODUCTION

Portal vein thrombosis (PVT) is a significant postoperative complication in patients with portal hypertension, and procedures such as devascularization, portal systemic shunt and liver transplantation can cause PVT[1-3]. As the degree of obstruction and location vary greatly, the clinical manifestations of PVT are also highly variable[4-6]. Depending on the severity of the condition, it may even result in death of patients.

The incidence of PVT after portal hypertension surgery ranges from 22.2%-37.5%[1,7], depending on the surgical techniques used and patients selected. In China, the most common cause for portal hypertension is liver cirrhosis due to hepatitis B. There is an increasing trend in PVT after portal hypertension surgery for hepatitis B liver cirrhosis.

Currently, diagnosis of portal vein thrombosis is mainly based on imaging studies[8-10], especially colour Doppler ultrasound, because of its ability to provide not only information on blood vessel location, blood flow rate and direction, but also haemodynamic information, which aids diagnosis accuracy[11,12]. It is able to provide information on the thrombosed veins via echo analysis, detect the presence of post-stenotic dilatation, as well as flow defects and turbulence due to venous thrombosis[13,14]. Although portal vein thrombosis can be accurately diagnosed using this method, it produces side effects simultaneously. Therefore, the focus of clinicians is to prevent portal vein thrombosis after portal hypertension surgery as early as possible, the early predictive indicator of postoperative PVT is still lacking.

Currently, clinicians use the incrteased platelet count as the main deciding factor for initiating prophylactic treatment, but the prognostic value of platelet count in PVT is debatable[15,16]. Some studies showed that even at the time of portal vein thrombosis formation, the level of platelet count has not yet reached 500×10^9/L, and patients showing increased levels of platelet count do not

have portal vein thrombosis[17]. Hence, the question of the indicator for initiation of preventive and therapeatic measures is still debatable.

Clotting factors are important in the assessment of liver function and have certain clinical significance, but have yet to show a good clinical value as a diagnostic indicator for thrombotic diseases. At present, D-dimer, the molecular marker in the fibrinolysis cycle, has shown a good negative and positive predictive value in thrombotic diseases such as deep vein thrombosis in the lower limbs and pulmonary embolism, and has therefore been used in studies on its clinical application[18,19]. However, since patients with portal hypertension due to liver cirrhosis have innate clotting and fibrinolytic disorders[20,21], more studies are required to show whether D-dimer has a better predictive value PVT after portal hypertension surgery due to the combined effects of portal vein thrombosis on platelets, clotting and fibrinolysis[22].

MATERIALS AND METHODS

Patient selection criteria

Patients who were admitted to the Third Affiliated Hospital of the Sun Yat-Sen University from September 2004 to March 2006, and diagnosed with portal hypertension due to liver cirrhosis (hepatitis B) were included in this study. The patients with splenomegalia and hypersplenia underwent splenectomy, and the patients with esophageal variceal bleeding underwent splenectomy with gastroesophageal devascularization, or with endoscopic variceal ligation (EVL). Patients who underwent portal hypertension shunting surgery, liver transplant patients, and patients having concurrent liver cirrhosis and hepatocellular carcinoma, and patients diagnosed with PVT before surgery, were excluded from this study.

General demographics

A total of 52 patients (46 males and 6 females) fulfilled the selection criteria. Their age ranged from 20-60 years, with a median age of 46 years (Table 1). Surgical procedures performed on patients included splenectomy and gastroesophageal devascularization (Hassab's operation) as previously described[23]. In brief, an extended left subcostal incision or a L incision of the left upper abdomen was used for extreme splenomegaly. After routine splenectomy, the gastric branch and 5-8 small branches of the gastric coronary veins were disconnected. The esophageal branch was disconnected and suture-ligated. The gastric posterior vein was ligated by suturing, and the left subphrenic vein was ligated as well. In cases of splenectomy with EVL, after splenectomy, an endoscope (GIF 240 or 260, Olympus Optical, Tokyo) was introduced during operation. Ligation was carried out 6-12 times by placing a single rubber band (Bard Interventional Products, Tewksbury, Mass.) over a varix.

Diagnosis of PVT

When colour Doppler ultrasonography imaging showed changes consistent with thrombus formation in the main portal vein, right portal vein, right anterior branch, right posterior branch, left portal vein, left horizontal branch, left sagittal portion, splenic vein, proximal and distal mesenteric vein, PVT was confirmed to be a complication after surgery.

Table 1 Characteristics of 52 patients studied

		Thrombus	Non-thrombus	*P* value
Age (mean ± SD)		41.45 ± 10.83	44.3 ± 8.65	0.388
Gender	Male	15	31	
	Female	2	4	0.649
Child's grade	Grade A	6	12	
of Liver function	Grade B	10	21	0.997
	Grade C	1	2	
Surgical	Splenectomy	7	13	
procedure	Splenectomy + EVL[1]	9	19	0.923
	Splenectomy + ligation[2]	1	3	

[1]Refers to endoscopic variceal ligation; [2]Refers to gastroesophageal devascularization.

Measurement of D-dimer levels

Dynamic D-dimer changes in 52 patients were monitored perioperatively, and the portal vein patency and the incidence of PVT were detected routinely by color Doppler ultrasonography from 7 to 14 d postoperatively. The 52 patients were divided into thrombosis group and non-thrombosis group, depending on the occurrence of PVT after surgery. Peripheral venous blood was collected perioperatively. Three mL blood was centrifuged at 1000 r/min for 15 min before plasma was prepared for D-dimer detection. D-dimer was detected by latex-agglutination assay (semi-quantitative method), and the reagent was provided by Shanghai Sun Company, and the procedure was performed following the instructions provided with the reagent pack.

Statistical analysis

After normality tests, D-dimer indicators showing a skewed distribution were subjected to signed rank test between the thrombosis and non-thrombosis groups, using the difference in the median and interquartile range to illustrate the central and disperse tendency. Chi-square test was used to analyze the dispersion. Sensitivity, specificity, positive and negative predictive value were calculated. ROC curve was plotted. Statistical analysis was performed using the SPSS software version 13.0 for Windows.

RESULTS

Characteristics of the 52 patients studied

Of the 52 patients, 17 developed PVT after surgery, and the incidence of PVT 7-14 d after portal hypertension surgery was 33.69%. There was no significant statistical difference in age, gender, liver function and surgical procedure between the thrombosis and non-thrombosis groups (Table 1).

Table 2 D-dimer level in the thrombosis and non-thrombosis groups (μg/mL)

	Before surgery	Day 1 after surgery	Day 5 after surgery
Non-thrombus group	1 (0.5, 2)	4 (2, 8)[1]	4 (2, 4)[1,2]
Thrombus group	1 (0.5, 2)	4 (2, 8)[1]	8 (4, 8)[1,3]
Z value	1.863	0.318	3.264
P value	0.062	0.751	0.001

[1]Refers to the higher level that is statistically significant after surgery than that before surgery; [2]Refers to the lower level that is statistically significant before surgery than that one day after surgery; [3]Refers to the higher level that is statistically significant before surgery than that one day after surgery.

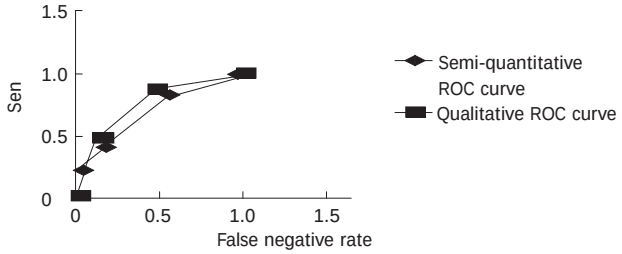

Figure 1 Semi-quantitative and qualititive ROC curves.

Perioperative D-dimer level

The D-dimer level was significantly higher in the thrombosis group than in the non-thrombus group on day 5 after surgery ($P = 0.001$) (Table 2).

Analysis of predictive value for D-dimer in PVT after surgery

By using the change in D-dimer level post-surgery (used as the standard) and the D-dimer (used as semi-quantitative standard) as the diagnostic standard for PVT in liver cirrhosis patients after portal hypertension surgery, we achieved 37.5% accuracy in predicting PVT incidence, which is consistent with the reported data[1]. The sensitivity (Sen), specificity (Spe), positive predictive value (PPV) and negative predictive value (NPV) are listed in Table 3. It can be seen from Table 3 that the positive predictive value was 66.7% for the D-dimer one day after surgery, and the negative predictive value for the continuous increasing was 77.5%, which was significantly higher than the semi-quantitative value of 61.65% for PPV and 70.83% for NPV. Under the extreme circumstances, the semi-quantitative value for PPV and NPV was 100%, respectively.

Based on the different changes in D-dimer levels and the false positive rates and sensitivity rates under different D-dimer levels, a ROC curve was plotted. The semi-quantitative and qualitative standards for ROC curve are shown in Figure 1. Using the ROC analysis, the area under the semi-qualitative ROC curve was Az = 0.794, $P = 0.000$, and the 95% confidence level was 0.794 ± 0.114. The area under the qualitative ROC curve was Az = 0.739, $P = 0.001$, and the 95% confidence level was 0.795 ± 0.146. Both standards had a median diagnostic value, and there was no statistical significant difference in the areas under the ROC curve ($Z = 0.586$, $P = 0.719$).

Table 3 Diagnostic value for different D-dimer diagnostic standards (%)

		Sensitivity	Specificity	PPV	NPV
Qualitative	No decrease	88.2	57.14	42.85	88.25
standard	Increase	47.06	88.57	66.67	77.50
Semi-quantitative	> 0.5	1	0	37.50	0
standard	> 1	1	0.05714	38.88	100
	> 2	0.8235	0.4858	49	82.00
	> 4	0.4118	0.8571	61.65	70.83
	> 8	0.2353	1	100.00	33.89
	> 16	0.2353	1	100.00	33.89

DISCUSSION

D-dimer is an antigenic determinant of fibrin, which, at the D-region, combines with factor XIIIa, and persists as an X-oligopolymer, as well as with other fibrin degradation products. However, it does not exist with profibrin breakdown products, and is a specific indicator of synthesis and breakdown of fibrin in the system. An increasing D-dimer level indicates active clotting and fibrinolysis in the system[24,25].

At present, D-dimer has a good negative and positive predictive value, which is used in the outpatient and emergency setting, to aid selective diagnosis of suspected thrombotic diseases[19,26-28]. Risch *et al*[29] reported that at D-dimer levels of 1 μg/mL and 10 μ/mL (VIDAS DD), the sensitivity is 83.3% and 23.8%, respectively, and the specificity is 65.8% and 98.7%, respectively. In our study, the D-dimer level was higher than the reported level in liver cirrhosis patients after portal hypertension surgery, measured at similar sensitivity conditions, but had a lower specificity than the reported one. This may be attributed to the hyperactive state of the coagulation pathway due to the high coagulability state in liver cirrhosis patients, activated fibrinolysis, blood products used perioperatively, and trauma due to surgery[20-22,30].

Dynamic D-dimer value monitoring during the early stages after portal hypertension surgery may be more useful in achieving a meaningful diagnosis of PVT due to thrombosis formation. By analyzing the D-dimer after surgery and its predictive value for PVT, our study proved that the positive predictive value was 100% at ⩾ 16 μg/mL (Table 3). From the ROC curve (Figure 1), we could see that the qualitative standard was situated towards the left upper side of the semi-quantitative standard ROC curve, showing a higher sensitivity, but a lower rate of false positive response. Under the two extreme ends, the semi-quantitative standard curve was on the left-hand-side of the qualitative ROC curve, suggesting that the former may have a better diagnostic value. Therefore, combining semi-quantitative and qualitative measurement may be the best option for diagnosing PVT in liver cirrhosis patients after portal hypertension surgery.

In conclusion, D-dimer is an early predictive indicator of PVT after portal hypertension surgery in hepatitis B virus-related cirrhosis. Dynamic D-dimer monitoring should be performed perioperationly, and if elevation of the D-dimer levels persists or exceeds 16 μg/mL, PVT may occur, and anti-coagulation prevention and treatment should be considered.

COMMENTS

Background

Portal vein thrombosis (PVT) is a significant complication after portal hypertension surgery due to liver cirrhosis. The focus of clinicians is to prevent it as early as possible, but we still lack the early predictive indicator of postoperative PVT.

Research frontiers

PVT is a significant postoperative complication in patients with portal hypertension, especially after splenectomy. Since the mechanism of postoperative PVT remains unknown, it is difficult to prevent postoperative PVT. Most studies indicate that local operation may be the factor for postoperative PVT. Some studies indicate that D-dimer may be helpful in the diagnosis of deep vein thrombosis of lower extremity.

Innovations and breakthroughs

At present, most researchers and doctors diagnose PVT *via* imageology, especially color Doppler ultrasonography, and some reports indicate that platelets may be the predictive factor for PVT. D-dimer is a good predictive factor for deep vein thrombosis of lower extremity. The results of our study indicate that D-dimer may be a predictive factor for PVT after portal hypertension surgery in hepatitis B virus-related cirrhosis.

Applications

Since D-dimer is a predictive factor for PVT, PVT and its fatal complications can be prevented. By combining imageology, we can institute a scheme for the management of PVT, which helps to understand the mechanism of PVT.

Terminology

D-dimer: known as fragment D-dimer, fibrin degradation fragment; PVT: portal vein thrombosis. ROC curve: a graphic means for assessing the ability of a screening test to discriminate between healthy and diseased persons.

Peer review

This is a well-conducted study. It describes another way for the predictive diagnosis of PVT, but the relationship between the mechanisms of PVT and D-dimer remains unknown. D-dimer is only of a predictive diagnostic value.

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RAPID COMMUNICATION

Combined treatment of hepatocellular carcinoma with partial splenic embolization and transcatheter hepatic arterial chemoembolization

Jin-Hua Huang, Fei Gao, Yang-Kui Gu, Wen-Quan Li, Lian-Wei Lu

Jin-Hua Huang, Fei Gao, Yang-Kui Gu, Wen-Quan Li, Lian-Wei Lu, Department of Imaging and Interventional Radiology, Cancer Center, Sun Yat-Sen University, Guangzhou 510060, Guangdong Province, China
Correspondence to: Jin-Hua Huang, Department of Imaging and Interventional Radiology, Cancer Center, Sun Yat-Sen University, Guangzhou 510060, Guangdong Province, China. hjinh@mail.sysu.edu.cn
Telephone: +86-20-87343272 Fax: +86-20-87343272
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Abstract

AIM: To prospectively evaluate the efficacy and safety of partial splenic embolization (PSE) combined with transcatheter hepatic arterial chemoembolization (TACE) in treatment of hepatocellular carcinoma (HCC).

METHODS: Fifty patients suffering from primary HCC associated with hypersplenism caused by cirrhosis were randomly assigned to 2 groups: group A receiving PSE combined with TACE (n = 26) and group B receiving TACE alone (n = 24). Follow-up examinations included calculation of peripheral blood cells (leukcytes, platelets and red blood cells) and treatment-associated complications.

RESULTS: Prior to treatment, there was no significant difference in sex, age, Child-Pugh grade, tumor diameter, mass pathology type and peripheral blood cell counts between the 2 groups. After treatment, leukocyte and platelet counts were significantly higher in group A during the 3-mo follow-up period (P < 0.05), but lower in group B (P < 0.05). Severe complications occurred in 3 patients (11.5%) of group A and in 19 patients (79.2%) of group B (P < 0.05), and there was no significant difference in symptoms of post-embolization syndrome, including abdominal pain, fever, mild nausea and vomiting between the 2 groups (P > 0.05).

CONCLUSION: PSE combined with TACE is more effective and safe than TACE alone for patients with HCC associated with hypersplenism caused by cirrhosis.

Key words: Hepatocellular carcinoma; Hypersplenism; Cirrhosis; Partial splenic embolization; Transcatheter hepatic arterial chemoembolization

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INTRODUCTION

Transcatheter hepatic arterial chemoembolization (TACE) has become the first choice of treatment for unresectable hepatocellular carcinoma (HCC)[1-4]. Since 70%-90% of HCC patients are associated with liver cirrhosis, portal hypertension and hypersplenism, treatment of HCC is usually affected by low peripheral blood cell counts (leukcytes, platelets and red blood cells) and high incidence of hemorrhagic complications due to treatment and/or portal hypertension[5-8]. Moreover, chemotherapeutics during TACE is another cause for low peripheral blood cell counts because of myelosuppression. Partial splenic embolization (PSE), which is thought to be an effective alternative to splenomegaly[9,10] because of its milder injury and fewer complications, has been widely used in treatment of leukocytopenia and thrombocytopenia caused by splenomegaly since the report of Maddison in 1973[11].

MATERIALS AND METHODS

Patients

From December 2002 to May 2006, 50 consecutive patients with HCC associated with hypersplenism caused by liver cirrhosis and portal hypertension were enrolled in this study. The diagnosis of HCC was established on the basis of clinical laboratory data, computed tomography and biopsy. The diagnosis of hypersplenism and splenomegaly was made in the light of clinical laboratory data and computed tomography. The enrolling criteria for this study were patients with splenomegaly and thrombocytopenia (platelet count $\leqslant 60 \times 10^9$/L) and/or leukocytopenia (leukocyte count $\leqslant 3.0 \times 10^9$/L). Adequate supporting therapies were performed for patients having severe peritonealgia before treatment with PSE and TACE or TACE alone in order to decrease the amount of ascites. Patients meeting the above criteria were randomly assigned to either group A or group B based on the computer-generated randomization sequences. Of the 50 patients,

Table 1 Demographic, clinical, histological and laboratory characteristics of patients *n* (%)

Characteristics	Group A, *n*	Group B	*P*-value
Patients	26	24	
Sex			
Male	19 (73)	18 (75)	0.877[1]
Female	7 (27)	6 (25)	
Age (yr)	44.1 ± 12.1	45.0 ± 9.0	0 .760[2]
Child-Pugh grade			
A	2 (8)	2 (8)	
B	20 (77)	19 (79)	0.806[1]
C	4 (15)	3 (13)	
Pathology type			
Mass type	14 (54)	14 (58)	0.834[1]
Node type	10 (38)	8 (33)	
Diffusion type	2 (8)	2 (8)	
Tumor diameter (cm)			
Peripheral blood cell counts	4.64 ± 2.34	4.44 ± 2.58	0.780[2]
WBC ($\times 10^9$)	2.45 ± 0.41	2.40 ± 0.51	0.734[2]
PLT ($\times 10^9$)	45.95 ± 9.49	45.02 ± 8.96	0.723[2]
RBC ($\times 10^{12}$)	3.02 ± 0.49	3.07 ± 0.51	0.750[2]

[1]Data are determined with the χ^2 test; [2]Data are determined with the *t*-test.

26 received PSE in combination with TACE (group A), 24 received TACE alone (group B).The characteristics of these patients are summarized in Table 1.

Methods

The patients in group A were treated with PSE and TACE, first with PSE, and then with TACE, while the patients in group B received TACE alone.

PSE was performed as follows. In brief, a 5.0 French catheter (Terumo, Tokyo, Japan) was inserted into the femoral artery by the Seldinger method, celiac angiography and selective splenic arterial angiography were routinely performed to observe the distribution of splenic arteries and collateral circulation routes (Figure 1A), the tip of the catheter was placed as distal as possible at the hilus of the spleen, and embolization was performed using gelfoam particles (1-2 mm) suspended in an antibiotic solution (16 mg gentamicin sulfate) and contrast medium. The extent of embolization was set at 50%-70%. To achieve this, embolization was performed progressively by means of repeated injections of gelfoam particles under angiography control. Immediate angiography was done after each injection and the extent of embolization was expressed as the percentage of the ablated splenic parenchyma area shown by post-embolization angiography against the total splenic parenchyma area given by pre-embolization angiography. When a 50%-70% ablation of the splenic parenchyma was obtained (Figure 1B), the embolization was terminated. The extent of embolization was simultaneously estimated on angiography. In case the two estimates failed to agree, the mean value was taken. Finally, the precise extent of embolization was determined by CT scan 2 wk later.

Under strict conditions, TACE was performed as follows. In brief, a 5.0 French catheter (Terumo, Tokyo, Japan) was inserted into the femoral artery with the Seldinger method, celiac angiography and selective hepatic arterial angiography were routinely performed to observe the tumor blood-supply, distribution of hepatic arteries and collateral circulation routes (Figure 1C), the tip of the catheter was placed at the feeding artery of the tumor, and embolization was performed using an emulsion mixture of lipoidal ultra-fluid (Guerbet, France), perarubicin (50 mg/m^2) and DDP (80 mg/m^2). The maximum dose for embolization was based on the size of the tumor, blood supply and hepatic function of the patient. When the tumor was filled well with emulsifier, the embolization was terminated (Figure 1D).

Follow-up protocol

All patients underwent abdominal CT scanning (Light Speed QX/I CT scanner, GE Medical Systems, Milwaukee, Wis) 1 wk before operation (Figure 1E). Patients in group A also underwent abdominal CT scanning (Light Speed QX/L CT scanner,GE Medical Systems,Milwaukee,Wis) 2 wk after PSE/TACE treatment (Figure 1F). The extent of embolization (%) was determined by dividing the infarction volume, which is the whole splenic volume minus the residual splenic volume, by the whole splenic volume based on the CT examination 2 wk after PSE/TACE treatment.

After treatment with PSE in combination with TACE or with TACE alone, all patients remained in the hospital with their severe complications observed and were then followed up at the Outpatient Clinic. Peripheral blood cell parameters including white blood cells (WBC), platelets (PLT) and red blood cells (RBC) in group A after PSE/TACE treatment and in group B after TACE treatment were respectively monitored during the 1-wk, 2-wk, 1-mo, 2-mo and 3-mo follow-up after PSE/TACE treatment.

Statistical analysis

All data were analyzed using the SAS software (Vertion 8.1, SAS Institute, Cary, NC). Significance was established at $P < 0.05$. To determine statistically significant difference between the two groups, the *t*-test or the χ^2 test was used. The paired *t*-test was used to determine the difference in group A before and after PSE/TACE treatment and in group B before and after TACE treatment, and between groups A and B after treatment.

RESULTS

Chronological changes in peripheral blood cell counts

No significant difference was found in sex, age, Child-Pugh grade, tumor diameter, mass pathology type and peripheral blood cell counts between the 2 groups (Table 1). The peripheral blood cell counts before PSE/TACE or TACE treatment and from the third day to the forth week after PSE treatment are listed in Tables 2-4. There were no significant differences in WBC, PLT and RBC counts between the 2 groups before PSE/TACE or TACE treatment ($P > 0.05$). There were significant differences in WBC and PLT counts before and after PSE/TACE treatment ($P < 0.001$, Tables 2 and 3). WBC and PLT counts were significantly higher from the first week to the third month after PSE/TACE treatment. There were significant differences in WBC and PLT counts before and

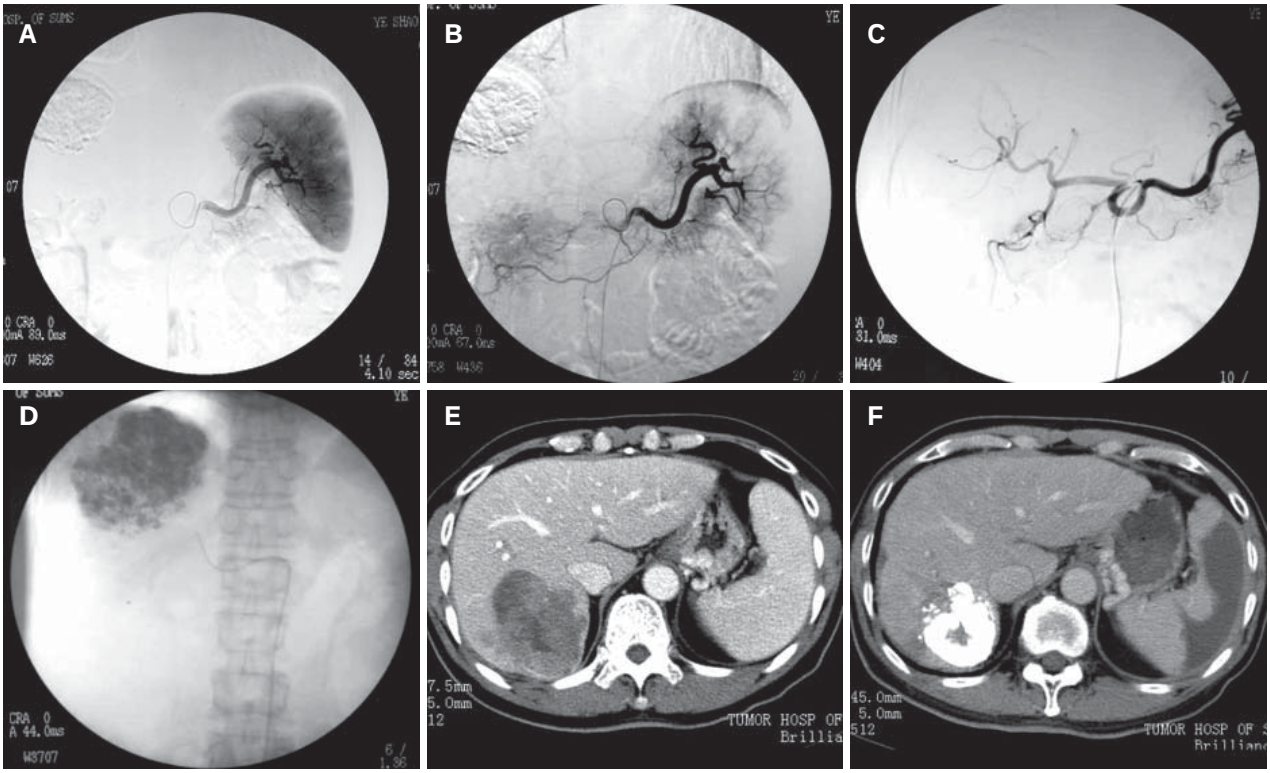

Figure 1 PSE treatment for a 68-year-old male case of HCC with splenomegaly and thrombocytopenia. **A**: Splenic arteriography before PSE showing the whole splenic parnchymal image; **B**: Splenic arteriography after PSE showing the residual splenic parnchymal image, part of the peripheral splenic parenchyma was ablated, and the extent of embolization was roughly estimated of approximately 60%; **C**: Celiac arteriography before TACE showing the tumor blood-supply image; **D**: TACE is terminated when the tumor is filled with emulsifier; **E**: Transverse CT image revealing splenomegaly at 1 wk before PSE/TACE; **F**: Transverse CT image revealing the infarction of peripheral splenic parenchyma at 2 wk after PSE. The extent of embolization was 62% calculated by CT volume analysis software.

Table 2 Follow-up results of WBC counts ($\times 10^9$/L)

Time	Group A		Group B		*P*-value[2]
	mean ± SD	*P*-value[1]	mean ± SD	*P*-value[1]	
Pre-treatment	2.45 ± 0.41		2.40 ± 0.51		0.734
Post-treatment					
1 wk	7.26 ± 0.96	< 0.001	1.77 ± 0.38	< 0.001	< 0.001
2 wk	6.42 ± 1.02	< 0.001	1.68 ± 0.39	< 0.001	< 0.001
1 mo	6.31 ± 0.83	< 0.001	1.72 ± 0.65	< 0.001	< 0.001
2 mo	6.03 ± 0.93	< 0.001	1.91 ± 0.73	0.0032	< 0.001
3 mo	5.36 ± 0.64	< 0.001	2.02 ± 0.48	0.013	< 0.001

[1]Comparison of WBC counts before and after treatment at different time points within each group; [2]Comparison of WBC counts between the two groups at different time points determined with *t*-test.

Table 3 Follow-up results of PLT counts ($\times 10^9$/L)

Time	Group A		Group B		*P*-value[2]
	mean ± SD	*P*-value[1]	mean ± SD	*P*-value[1]	
Pre-treatment	45.95 ± 9.49		45.02 ± 8.96		0.723
Post-treatment					
1 wk	169.21 ± 26.55	< 0.001	28.56 ± 5.11	< 0.001	< 0.001
2 wk	136.50 ± 13.12	< 0.001	26.62 ± 7.31	< 0.001	< 0.001
1 mo	133.46 ± 16.21	< 0.001	27.46 ± 6.29	< 0.001	< 0.001
2 mo	125.73 ± 18.35	< 0.001	31.06 ± 6.70	< 0.001	< 0.001
3 mo	119.86 ± 12.43	< 0.001	33.15 ± 6.91	< 0.001	< 0.001

[1]Comparison of PLT counts before and after treatment at different time points within each group; [2]Comparison of PLT counts between the two groups at different time points determined with *t*-test.

after TACE treatment in group B ($P < 0.05$, Tables 2 and 3). WBC and PLT counts were significantly lower in group B from the first week to the third month after TACE treatment. There were significant differences in WBC and PLT counts between groups A and B ($P < 0.001$, Tables 2 and 3). WBC and PLT counts were significantly higher in group A after PSE/TACE treatment than in group B from the first week to the third month after TACE treatment. However, there were no significant differences in RBC counts between the 2 groups ($P > 0.05$, Table 4).

Complications

Symptoms of post-embolization syndrome, including abdominal pain, fever and mild nausea and vomiting, occurred in our patients. Abdominal pain was found in 76.9% (20/26) patients of group A and was alleviated by durogesic or oxycodone, in 75.0% (18/24) patients of group B and was alleviated by Tramadol with no significant differences between the two groups. The incidence of fever was 84.6% (22/26) in group A and was lowered by dexamethasone, 83.3% (20/24) in group B and was lowered by salicylic acid drugs with no significant differences between the two groups. The incidence of mild nausea and vomiting was 19.2% (20/26) in group A, 25.0% (6/24) in group B with no significant differences between the two groups. Severe complications occurred in 3 patients (11.5%) of group A, in 19 patients (79.2%) of group B (Table 5). A large amount of pleural effusion

Table 4 Follow-up results of RBC counts ($\times 10^{12}$/L)

Time	Group A		Group B		*P*-value[2]
	mean ± SD	*P*-value[1]	mean ± SD	*P*-value[1]	
Pre-treatment	3.02 ± 0.49		3.07 ± 0.51		0.75
Post-treatment					
1 wk	2.84 ± 0.72	0.297	2.93 ± 0.56	0.375	0.639
2 wk	2.88 ± 0.54	0.325	2.97 ± 0.68	0.583	0.606
1 mo	2.81 ± 0.36	0.073	2.93 ± 0.71	0.442	0.430
2 mo	2.92 ± 0.42	0.418	2.85 ± 0.62	0.185	0.623
3 mo	3.04 ± 0.50	0.924	2.97 ± 0.47	0.439	0.644

[1]Comparison of RBC counts before and after treatment at different time points within each group; [2]Comparison of RBC counts between the two groups at different time points determined with *t*-test.

Table 5 Complications observed in 50 patients 2 wk after treatment *n* (%)

Complications	Group A	Group B	*P*-value
Abdominal pain	20 (76.9)	18 (75.0)	0.874[1]
Fever	22 (84.6)	20 (83.3)	0.903[1]
Mild nausea and vomiting	5 (19.2)	6 (25.0)	0.623[1]
Large amount of pleural effusion or ascites	1 (3.9)	6 (29.2)	0.016[1]
Bacterial peritonitis	1 (3.9)	6 (25.0)	0.033[1]
Variceal bleeding	1 (3.9)	7 (29.2)	0.016[1]

[1]Data are determined with the χ^2 test.

and ascites was found in 1 patient of group A and in 6 patients of group B, leading to dyspnea or abdominal pain which was resolved by thoracentesis and paracentesis. Bacterial peritonitis occurred in 1 patient of group A and in 6 patients of group B 1 mo after PSE treatment. Variceal bleeding was observed in 1 patient of group A and in 7 patients of group B and was controlled by conservative therapy. There were significant differences in severe complications between the 2 groups ($P < 0.05$, Table 5). The occurrence of severe complications such as pleural effusion or ascites, bacterial peritonitis and variceal bleeding was significantly higher in group B than in group A after treatment.

DISCUSSION

HCC is often associated with hypersplenism due to liver cirrhosis. In such cases, it is very difficult to perform TACE because of the high incidence of hemorrhagic complications and/or portal hypertension, as well as poor tolerance of cirrhotic patients to chemotherapeutic drugs[12]. PSE is a useful support therapy for portal hypertension or hypersplenism and has taken the place of surgical splenectomy[9,10,13,14]. PSE appears to be effective in reducing episodes of variceal bleeding, improving hematologic parameters, enhancing hepatic protein synthesis, and reducing the severity of hepatic encephalopathy[15-18]. Roversi *et al*[12] reported that complications such as pleural effusion or ascites, bacterial peritonitis and variceal bleeding occurred in six patients with nodular HCC and cirrhosis (Child B) after treated with TACE in combination with PSE. In our study, thrombocytes, leucocytes and erythrocytes increased markedly, severe complications occurred in 3 patients (11.5%) of group A and in14 patients (79.2%) of group B. N'Kontchou *et al*[19] showed that severe complications occurred in six patients (16%) in their study, namely transient ascites in 2, splenic and/or portal vein thrombosis in 2, and splenic abscess in 2. Sakai *et al*[20] observed two cases suffering from severe complications after PSE treatment in 17 patients with cirrhosis. Other severe complications of PSE treatment such as pleural effusion, rupture of spleen, portal vein thrombosis have also been reported[13,21,22].

In this study, embolization ranged from 50% to 70%. Lee *et al*[23] reported that there are significant differences in platelet values between low and high embolization areas in patients with cirrhosis. The complication rate for < 30% and > or = 30% embolization areas is 50% and 100%, respectively. In our study, severe complications had a close relationship with the extent of embolization of the spleen. Among the 4 patients with an embolization of over 70%, 3(75%) developed severe complications. On the contrary, among the 22 patients with embolization of 70% or lower, only 1 (5%) developed severe complications, suggesting that PSE should be strictly limited to less than 70% of the splenic volume in order to reduce severe complications.

Gelfoam particles are the most commonly used embolic material in PSE [9,10,13,14,24-26], and extensive research has confirmed the short- or long-term efficacy of PSE using gelfoam particles as embolic material[9,13,14,25]. N' Kontchou *et al*[19] also performed PSE using PVA particles as embolic material (200-1000 μm in diameter) in patients with cirrhosis, but the efficacy and safety were uncertain, especially the long-term efficacy in peripheral blood cell count and safety. In this study, we used gelfoam particles as embolic material in PSE and achieved good results, indicating that gelfoam particles are safe materials in PSE.

In conclusion, combined one-step TACE/PSE treatment can improve the tolerance of HCC patients with advanced/decompensated cirrhosis and hypersplenism to chemotherapeutic drugs and reduce the risk of complications of invasive radiologic procedures and/or portal hypertension. PSE may resolve cytopenia and clinical complications related to hypersplenism or splenomegaly. However, due to severe complications, particularly splenic abscess, the indications for PSE should be limited and the extent of necrosis should be controlled during the PSE procedure.

COMMENTS

Background

In many cases of hepatocellular carcinoma (HCC) associated with liver cirrhosis and hypersplenism, it is very difficult to perform TACE because of the high incidence of hemorrhagic complications and poor tolerance of patients to chemotherapeutic drugs. The combined one-step TACE/PSE treatment can improve the tolerance of patients to chemotherapeutic drugs and reduce hemorrhagic complications of invasive radiologic procedures and/or portal hypertension.

Research frontiers

In this study, hematologic parameters and severe complications such as pleural effusion or ascites, bacterial peritonitis and variceal bleeding were observed. PSE may resolve cytopenia and clinical complications related to hypersplenism

or splenomegaly. Embolization and embolic material in PSE have not been standardized, but in our study, 50% to 70% of embolization was achieved with gelfoam particles as embolic material.

Innovations and breakthroughs

TACE has become the best choice of treatment for unresectable HCC. PSE may resolve cytopenia and clinical complications related to hypersplenism or splenomegaly. However, there have been few reports on the feasibility and effects of the combined one-step TACE/PSE treatment in cases of HCC associated with liver cirrhosis and hypersplenism.

Applications

Based on the results of our study, PSE in combination with TACE is more effective and safer for patients with HCC associated with hypersplenism caused by cirrhosis than TACE alone.

Terminology

TACE, an abbreviation of transcatheter hepatic arterial chemoembolization, is now widely used in treatment of HCC. PSE means partial splenic embolization.

Peer review

This paper provides some information about combining splenic embolization with TACE for gastroenterologists, hepatologists, and interventional radiologists.

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RAPID COMMUNICATION

Surgical management in biliary restricture after Roux-en-Y hepaticojejunostomy for bile duct injury

Ji-Qi Yan, Cheng-Hong Peng, Jia-Zeng Ding, Wei-Ping Yang, Guang-Wen Zhou, Yong-Jun Chen, Zong-Yuan Tao, Hong-Wei Li

Ji-Qi Yan, Cheng-Hong Peng, Jia-Zeng Ding, Wei-Ping Yang, Guang-Wen Zhou, Yong-Jun Chen, Zong-Yuan Tao, Hong-Wei Li, Department of Surgery, Ruijin Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200025, China
Correspondence to: Cheng-Hong Peng, Department of Surgery, Ruijin Hospital, School of Medicine, Shanghai Jiaotong University, 197 Ruijin Road, Shanghai 200025, China. chhpeng@188.com
Telephone: +86-21-64370045 Fax: +86-21-64333548
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Abstract

AIM: To discuss the surgical method and skill of biliary restricture after Roux-en-Y hepaticojejunostomy for bile duct injury.

METHODS: From November 2005 to December 2006, eight patients with biliary restricture after Roux-en-Y hepaticojejunostomy for bile duct injury were admitted to our hospital. Their clinical data were analyzed retrospectively.

RESULTS: Bile duct injury was caused by cholecystectomy in the eight cases, including seven cases with laparoscopic cholecystectomy and one with mini-incision choleystectomy. According to the classification of Strasberg, type E1 injury was found in one patient, type E2 injury in three, type E3 injury in two and type E4 injury in two patients. Both of the type E4 injury patients also had a vascular lesion of the hepatic artery. Six patients received Roux-en-Y hepaticojejunostomy for the second time, and one of them who had type E4 injury with the right hepatic artery disruption received right hepatectomy afterward. One patient who had type E4 injury with the proper hepatic artery lesion underwent liver transplantation, and the remaining one with type E3 injury received external biliary drainage. All the patients recovered fairly well postoperatively.

CONCLUSION: Roux-en-Y hepaticojejunostomy is still the main approach for such failed surgical cases with bile duct injury. Special attention should be paid to concomitant vascular injury in these cases. The optimal timing and meticulous and excellent skills are essential to the success in this surgery.

Key words: Bile duct injury; Biliary stricture; Roux-en-Y hepaticojejunostomy; Hepatic artery injury

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INTRODUCTION

Since its introduction in the 1990s by Dubois[1], laparoscopic cholecystectomy has become the "gold standard" treatment for symptomatic gallbladder stone disease. Limited postoperative discomfort, shorter hospitalization, and rapid postoperative recovery have been proven to be advantageous of the procedure. Concomitantly, it became obvious that the incidence of bile duct injury rose from 0.06% to 0.3%, as known for open cholecystectomy, to 0.5% to 1.4% when performed laparoscopically[2-6]. Bile duct injury following cholecystectomy is an iatrogenic catastrophe associated with significant perioperative morbidity and mortality[7,8], reduced long-term survival and quality of life[9,10], and high rates of subsequent litigation. Apparently, it is a great surgical challenge to handle with biliary restricture after Roux-en-Y hepaticojejunostomy for bile duct injury. The operation can be much more complex and difficult when compared with the first attempt for bile duct injury reparation.

MATERIALS AND METHODS

Methods

From November 2005 to December 2006, eight cases of biliary restricture after Roux-en-Y hepaticojejunostomy, performed at other hospitals for bile duct injury, were admitted to our hospital. The average age of those patients, seven female and one male, was 48.9 ± 7.5 (35-60) years. Bile duct injuries were all caused by cholecystectomy in other hospitals, including seven cases with laparoscopic cholecystectomy and one case with mini-incision choleystectomy. Although bile duct injury in three patients was initially treated by a T-tube placement within a choledocho-choledochostomy, Roux-en-Y hepaticojejunostomy was performed afterward (with a range of 4-13 mo) in those patients because of failure

Table 1 Patient basic status of bile duct injury

Patient number	Type of injury (Strasberg)	Combined with vascular injury	Operation type	Postoperative liver function
1	E1	-	Roux-en-Y	Normal
2	E2	-	Roux-en-Y	Normal
3	E2	-	Roux-en-Y	Normal
4	E2	-	Roux-en-Y	Normal
5	E3	-	Roux-en-Y	Normal
6	E3	-	External biliary drainage	TBIL among 100 mmol/L
7	E4	right HA injury	Roux-en-Y, right hepatectomy afterward	Normal
8	E4	Proper HA injury	Liver transplantation	Normal

HA: Hepatic artery; Roux-en-Y: Roux-en-Y hepaticojejunostomy; TBIL: Total bilirubin.

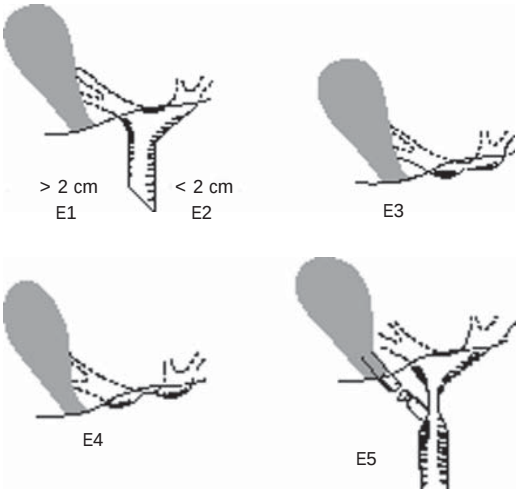

Figure 1 Strasberg classification of bile duct injury. E1: Transected main bile duct with a stricture more than 2 cm from the hilus; E2: Transected main bile duct with a stricture less than 2 cm from the hilus; E3: Stricture of the hilus with right and left ducts in communication; E4: Stricture of the hilus with separation of right and left ducts; E5: Stricture of the main bile duct and the right posterior sectoral duct.

in primary reparation. For the remaining five patients, Roux-en-Y hepaticojejunostomy was performed initially either during the operation of cholecystectomy or within a week after cholecystectomy. Unfortunately, all patients developed biliary restricture during the follow-up period, and were therefore transferred to our hospital.

Bile duct injury was classified according to Strasberg[11] when the medical files from the referring hospital were reviewed. After admission, those patients received abdominal CT and MRCP examination. Type E1 injury was found in one patient, type E2 injury in three, type E3 injury in two and type E4 injury in two patients (Table 1 and Figure 1). Both of the patients with type E4 injury also had a vascular lesion of the hepatic artery, with disruption of either the right hepatic artery or the proper hepatic artery. The average period between the primary Roux-en-Y hepaticojejunostomy and the operation in our hospital was 41.3 ± 46.5 (5-148) mo. Six of the eight patients received Roux-en-Y hepaticojejunostomy for the second time without stent implantation, and one of the six patients who had type E4 injury with the right hepatic artery disruption underwent right hepatectomy afterward. One patient who had type E4 injury with the proper hepatic artery lesion received liver transplantation, and the remaining one patient with type E3 injury received external biliary drainage.

RESULTS

All patients recovered uneventfully, and no significant complications occurred postoperatively. Three patients received additional or alternative surgical procedures. In one patient who had type E4 injury with the right hepatic artery disruption, the primary bile-enteric anastomosis of the left hepatic duct was fairly good, however the scarred biliary stricture occurred in the primary biliary-enteric anastomosis of the right hepatic duct, and multiple small abscesses were located in the right lobe of the liver. Considering the possibility of liver failure caused by insufficient remnant liver volume, we did not perform right hemihepatectomy at that time. Carefully removing those

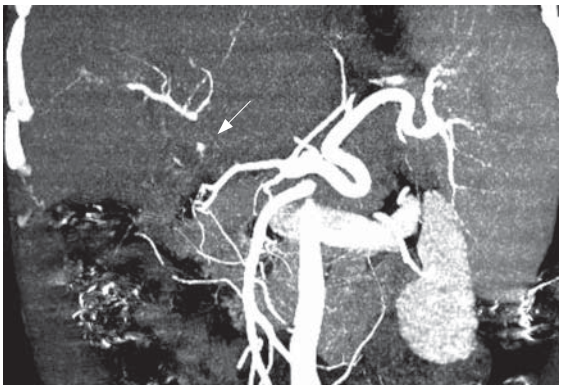

Figure 2 Computed Tomography angiography (CTA) of one type E4 injury patient displayed the lesion of the proper hepatic artery (arrow), although some compensatory collateral arterial blood supply from the left gastric artery could be identified. This patient received liver transplantation.

inflammatory scar tissues, we identified the openings of the right anterior hepatic duct and the right posterior hepatic duct. We connected these two bile duct openings into one by plastic reconstruction and performed biliary-enteric reanastomosis. Six weeks later, the abscess was found in the right hepatic lobe of this patient on CT with clinical symptoms. However, the left hepatic lobe compensatory enlarged and liver function improved, and thus the right hemihepatectomy was performed safely afterward. The other patient with type E4 injury had proper hepatic artery disruption, although compensatory collateral arterial blood supply from the left gastric artery could be identified by CT (Figure 2). The patient presenting secondary biliary cirrhosis combined with portal hypertension at admission received liver transplantation. The remaining patient with type E3 injury suffered from severe biliary infection, and the general condition was extremely poor. Although several stones were removed in the biliary-enteric anastomosis of the left duct during the operation, we could not expose

the main opening of the right hepatic duct even when painstakingly dissecting hepatic hilar tissue about 3 cm in depth. As a result, external biliary drainage had to be carried out.

At a median follow-up of 10 (range 4-17) mo, the six patients who received Roux-en-Y hepaticojejunostomy for the second time and one undergoing liver transplantation was clinically and biochemically stable without any pathologic findings. The symptoms of biliary infection of the patient receiving external biliary drainage were controlled, the liver function was ameliorated, and total bilirubin dropped from the preoperative level of 318 μmol/L to a level of around 100 μmol/L. This patient was waiting for liver transplantation.

DISCUSSION

In the United States and Canada, 34%-49% of surgeons have experienced a major bile duct injury, in one or two cases[5,12]. Increasing evidence has suggested that such injury should be managed by an experienced hepatobiliary surgeon[13] and the early recognition of injury directly affects the outcome[7]. Patients treated by the injuring surgeons have an increased death risk of 11% at nine years[14], yet in North America 58%-75% of injuries are still repaired by the injuring surgeons[9,12]. Similarly, such situation also occur in China. Undoubtedly, it is a surgical challenge to handle the failed cases of Roux-en-Y hepaticojejunostomy for bile duct injury.

Preoperative preparation

Initial treatment should focus on resuscitation of the patient, drainage of any collections to create a controlled enterocutaneous fistula and treatment of sepsis. Any unuseful intra-abdominal drains may be withdrawn subsequently from the hilum, reducing the inflammation caused by such a foreign body, thereby allowing the tissue to mature. Nutritional supports should be maintained during the whole perioperative period[15], since bile duct injury may even result in a systemic inflammatory response, with subsequent development of multiorgan failure. A low serum level of albumin at the time of surgery is often associated with a poor outcome[16]. Thus, it is important to address any nutritional deficit with enteral feeding, as long periods of biliary-enteric discontinuity will impair the function of the intestinal barrier and increase the risk of endotoxaemia and fat soluble vitamin deficiency[17].

Choice of operative method

If the biliary confluence is intact and there is no associated vascular injury, a hepaticojejunostomy onto the extrahepatic bile duct gives the best result[15,18-20]. Based on our preliminary experience, the number and diameter of bile duct openings at the hilum is not the limiting factor of the surgery. Murr *et al*[20] reported a 91% success rate and an 88% 5-year stricture-free survival. In liver transplantion surgery, biliary complications are almost universal following hepatic artery thrombosis. Vascular injuries contribute significantly to postoperative morbidity and mortality, particularly in cases of delayed diagnosis[21]. It has been shown that ductal ischemia due to concomitant hepatic arterial damage may be a cause of failed primary hepatojejunostomal reconstruction or late peripheral bile duct stenosis[22,23]. Occlusion of the right hepatic artery can lead to necrosis of the right hepatic lobe; therefore, it may be appropriate to consider a right hemihepatectomy[21]. In the case of poor general condition and uncontrollable biliary system infection, external biliary drainage might be the unique choice at the time of emergency. Patients who have developed secondary biliary cirrhosis should be considered to be the candidates for liver transplantation rather than further reconstruction, especially if there is significant portal hypertension[15]. Bile duct injury associated with complex vascular lesions might even necessitate liver transplantation[24].

Exposure of proximal bile duct

It is extremely important to create the biliary-enteric anastomosis to a healthy, non-inflamed, non-scarred duct. After the failure of the first attempt of Roux-en-Y hepaticojejunostomy, such dissection would become particularly difficult, for the level of scarred biliary stricture could be much higher than that of the primary bile duct injury. Once all adhesions of the right upper quadrant are sectioned, dissection of the jejunal limb is perfomed because misplacement and erroneous construction of Roux-en-Y are found in some patients[25]. For a correct hilar dissection, it is necessary to avoid the interruption of arterial branches as much as possible. No effort is given to completely dissect the arterial supply to the liver[26]. The hepatic artery itself and the previous surgical suture could be a useful anatomical mark in searching the bile duct at the hepatic hilum. The hilar plate is sectioned and the hilus is retracted caudally. If bile leak is observed during dissection, fine bile dilators are carefully inserted to identify the main ducts. Once the bile duct is explored, the scarred duct has to be removed up to a level at which a healthy duct is found. If the bifurcation is lost, with the isolated left and right hepatic duct, or the confluence is high and deep in the liver, dissection of the proximal bile ducts is not easily obtained. In such cases, partial liver resection of the segment Ⅳ and Ⅴ is done to allow adequate exposure of the left and right ducts, as described by Strasberg[27].

Surgical technique

In the present study, all patients received surgical repairs at their primary hospitals, and some even experienced numerous attempts. All those failed biliary-enteric anastomoses were placed at a too low level and exhibited technical faults such as the use of non-absorbable silk suture materials or a two-layer anstomotic technique. Attention to the anatomical placement of the anastomosis is of great importance[19,28,29], as failure after hepaticojejunostomy is usually caused by an anastomotic stricture, which is often ischemic in nature[28]. The healthy soft opening of the bile duct is crucial to the success of biliary-enteric reconstruction, however, the number and diameter of bile duct openings are comparatively of less importance. If bile duct openings at the hilum are nearby, plastic reconstruction could merge them into one or two openings (Figures 3 and 4). Although it is still controversial whether interrupted suturing or continuous

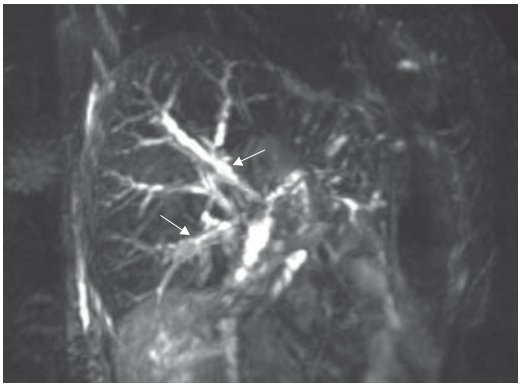

Figure 3 MRCP appearance of one type E3 injury patient showing dilation of intrahepatic bile ducts (arrows).

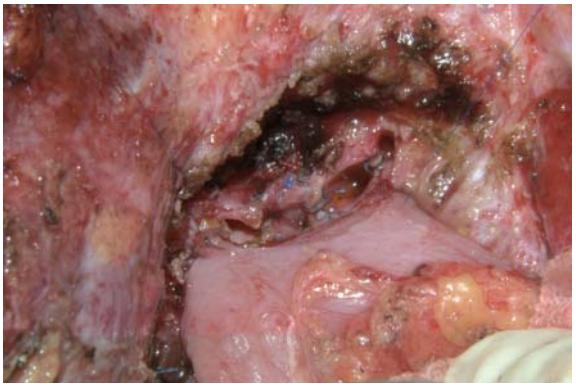

Figure 4 Plastic reconstruction in one type E3 patient merging several bile duct openings into one at the hilum after removal of the scarred ducts (the same patient in Figure 3).

running suturing is better for biliary-enteric anastomosis, we prefer the latter even in those extremely difficult cases with a single bile duct opening less than 3 mm in diameter. Actually, the distribution of tension in a continuous running suture would be more equal than an interrupted suture. Moreover, without the disturbance of multiple stitches as in interrupted suture, a continuous running suture would provide surgeons an easier way to focus their attentions to performing the anastomosis. The assistant surgeon should never pull the suture too tight during the entire procedure as we do expect to leave a 'growth factor' in every stitch. To improve the quality of biliary-enteric anastomosis is beyond the quarrel of the interrupted suture or continuous running suture. Based on our experience, traditional silk suturing should be abandoned in biliary-enteric anastomosis, since it might cause 'silk suture reactive stones,' looking like a necklace around the anastomosis area in some cases. Compared to most of the reported literature using absorbable PDS® suture for the anastomosis, we routinely use the non-absorbable Prolene® suture. Having observed a few cases of anastomotic edema of bile duct reconstruction using PDS® suture in previous liver transplanted patients, we consider that such absorbable suturing might be responsible for the foreign body reaction. However, this requires further investigations to draw a conclusion as to whether the absorbable PDS® suture or the non-absorbable Prolene® suture is advantageous in biliary-enteric anastomosis.

Attention to concomitant hepatic artery injury

It was reported that concomitant vascular injury was present in 71% of patients with Bismuth level Ⅳ lesions and in 63% of those with Bismuth level Ⅲ bile duct injury[23]. In our group, both of the type E4 injury patients had a hepatic artery lesion. Special attention should be paid to concomitant vascular injury in those complicated cases. The prognostic impact of vascular damage was underlined by Buell *et al*, who reported a 38% mortality rate in the presence of arterial lesions as against 3% in patients with injuries limited solely to the bile duct[30]. When vascular injury is recognized during the original operation, we would recommend immediate arterial reconstruction if the surgeon is capable or transfer the patient to a tertiary specialty department/clinic for definitive surgical repair. Delayed diagnosed vascular lesions are mostly not accessible for revascularization and can be followed by hepatic necrosis or persistent cholangitis resulting in end-stage liver cirrhosis. Based on our limited experience on one patient receiving liver transplantation and data reported by others, patients with bile duct injury associated with severe vascular lesion might be considered for liver transplantation.

Major bile duct injury during cholecystectomy is a disaster not only for the patient, but also for the surgeon. Although it would be extremely difficult to manage the patients with biliary restricture after Roux-en-Y hepaticojejunostomy for bile duct injury, good results could be achieved in the form of Roux-en-Y hepaticojejunostomy for the second time in those complicated cases. Undoubtedly, a better general preoperative condition, careful selection of the operation method, excellent surgical skills and meticulous postoperative management would account for a favourable outcome in bile duct injury cases.

COMMENTS

Background

Laparoscopic cholecystectomy has become the first choice of management for symptomatic cholecystolithiasis. While it is associated with decreased postoperative morbidity and mortality, bile duct injuries are reported more severe and more common when compared with open cholecystectomy.

Research frontiers

Bile duct injury would be aggravated by delayed recognition or a failed initial repair. It is a great challenge for surgeons to handle the biliary restricture after Roux-en-Y hepaticojejunostomy for bile duct injury. In most cases, if the biliary confluence is intact and there is no associated vascular injury, a secondary hepaticojejunostomy on the extrahepatic bile duct gives the best result.

Innovations and breakthroughs

It is extremely important to create the biliary-enteric anastomosis to a healthy, noninflamed, nonscarred duct, although the level of scarred biliary stricture could be much higher than that of the primary bile duct injury. Based on our preliminary experience, the number and diameter of bile duct openings at the hilum are not the limiting factors of the surgery. We prefer continuous running suture for biliary-enteric anastomosis.

Applications

Although it would be of great difficulty to manage the patients with biliary restricture

after Roux-en-Y hepaticojejunostomy for bile duct injury, good results could be achieved in a secondary Roux-en-Y hepaticojejunostomy in those complicated cases.

Terminology

Hepaticojejunostomy: anastomosis of hepatic duct and jejunum.

Peer review

The paper by Yan *et al* describes their surgical experience on biliary restricture after Roux-en-Y hepaticojejunostomy for bile duct injury in a Chinese university hospital. The results of the surgery seem to be impressive.

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CASE REPORT

Littoral-cell angioma of the spleen: A case report

Ángel Cosme, Ángel Tejada, Luis Bujanda, Manuel Vaquero, José Luis Elorza, Evelia Ojeda, Unai Goikoetxea

Ángel Cosme, Ángel Tejada, Luis Bujanda, Unai Goikoetxea, Department of Gastroenterology, Hospital Donostia, Spain
Manuel Vaquero, Department of Pathology, Hospital Donostia, Spain
Jose Luis Elorza, Departamet of Surgery, Hospital Donostia, Spain
Evelia Ojeda, Department of Internal Medicine, Hospital Donostia, Spain
Correspondence to: Ángel Cosme, Department of Gastroenterology, Hospital Donostia, Paseo Beguiristain, s/n, 20014, San Sebastián (Gipuzkoa), Spain. acosme@chdo.osakidetza.net
Telephone: +34-985-943003463 Fax: +34-985-943007065
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Abstract

Littoral-cell angioma (LCA) is a primary splenic vascular tumor that arises from the normal littoral cells lining the sinus channels of the splenic red pulp. We report a case of LCA of the spleen, which has been infrequently communicated in the literature. A 76-year-old man with a 2-wk history of weight loss, abdominal pain and changes in bowel habits was admitted to our hospital. Imaging studies (CT and MRI) showed multiple lesions in the spleen. Splenectomy was performed. Lining cells were positive for CD31/CD68 markers. Our case was associated with a serrated colonic adenoma. LCA is a benign vascular tumor of the spleen that needs to be included in the differential diagnosis of multiple splenic nodules.

Key words: Littoral-cell angioma; Spleen

Cosme Á, Tejada Á, Bujanda L, Vaquero M, Elorza JL, Ojeda E, Goikoetxea U. Littoral-cell angioma of the spleen: A case report. *World J Gastroenterol* 2007; 13(48): 6603-6604

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INTRODUCTION

Littoral-cell angioma (LCA) is a rare primary tumor of the spleen that was first described by Falk *et al* in 1991[1]. Considered a benign condition, this neoplasm arises from the red cell pulp sinuses and has intermediate features between those of endothelial and histiocytic cells. To the best of our knowledge, there have been 11 cases of LCA reported in the English-language literature since the description of this disease in 1991[2]. We report a case of LCA in a patient who had symptoms of weakness, pain and change in bowel habits. The combination of CT and MRI showed multiple lesions in the spleen. Postoperative pathology examination confirmed the final diagnosis of LCA.

CASE REPORT

A 76-year-old man was admitted to our hospital with a 2-wk history of weakness, weight loss, anorexia, hypogastric abdominal pain and change in bowel habits. His past medical history showed gastrectomy due to peptic ulcer at the age of 63 years and polypectomy of villous colonic adenoma 2 years before admission. Physical examination was normal. No splenomegaly was found. Results of routine laboratory tests were normal. Colonoscopy identified a polylobulated polyp $\geqslant$ 3 cm in size in the sigmoid colon, and polypectomy was performed. Pathology was conclusive for serrated adenoma. An enhanced CT scan of the abdomen showed multiple round, hypodense lesions in the spleen. Abdominal MRI revealed multiple splenic hypointensive lesions (Figure 1). Our presumptive preoperative diagnosis was lymphoma or hemangioma. Splenectomy was performed. Microscopically, lesions consisted of anastomosing vascular channels with papillary projections and cyst-like spaces. They were lined with endothelial cells that showed hemophagocytosis. Lining cells were positive for both vascular (CD31) and histiomonocytic (CD68) markers (Figure 2), but CD8 and CD3 were negative. Ten months after surgery, the patient was asymptomatic.

DISCUSSION

LCA of the spleen may occur at any age (1-77 years; median age, 50 years), with no sex-based predilection (female: male ratio, 5:3)[2,3]. Clinically, patients have splenomegaly, abdominal pain, pyrexia of unknown origin[4] or hypersplenism. LCA may present as an incidental finding. LCA may appear as single or multiple lesions in the spleen. Splenic lesions ranged from 0.2 to 6.0 cm. An extensive list of possibilities such as multiple hemangiomas, lymphoma, metastatic disease, and disseminated infections caused by fungi, mycobacteria, *Pneumocystis carinii* and *sarcoidosis*, should be considered in the differential diagnosis of multinodular splenomegaly. CT, MRI, US, and Tc99m RBC scan characteristics have been correlated with histological and immunohistochemical pathological features[5]. Consistent CT features of splenic LCA reported in the medical

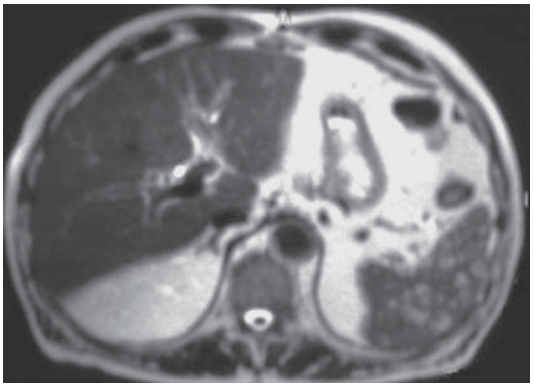

Figure 1 MRI: multiple hypointense lesions in the spleen (T_2).

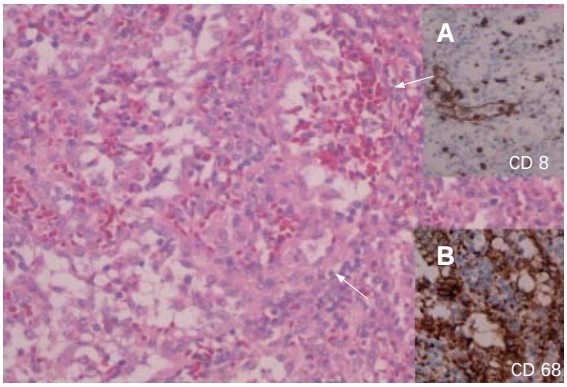

Figure 2 Vascular proliferation, well established (arrows), and very similar to normal spleen sinusoids. **A**: Negative for CD8; **B**: Positive for histiomonocytic markers.

literature include low-attenuating lesions on contrast-enhanced images[2,6]. Morphological diagnosis is based on the presence of anastomosing vascular channels lined with tall endothelial cells, focal papillary fronds, and normal splenic sinuses at the periphery of the lesion. Combination of morphological and immunohistochemical analyses that show a hybrid endothelial-histiocitic phenotype establish the diagnosis of LCA[1,7]. Two case reports have described variants of LCA with histological features of malignancy[8,9].

One third of the previously reported cases were associated with tumors of visceral organs, including colorectal, renal, hepatocellular[10], lung[11] and pancreatic adenocarcinomas, malignant lymphoma[12], myelodysplastic syndrome[13], or aplastic anaemia[14]. Since the malignant potential of LCA has not been firmly established in the literature, we recommend close clinical follow-up of patients with LCA of the spleen.

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CASE REPORT

Surgical intervention may not always be required in gossypiboma with intraluminal migration

H Alis, A Soylu, K Dolay, M Kalaycı, A Ciltas

H Alis, A Soylu, K Dolay, M Kalaycı, General Surgery Department Endoscopy Unit, Bakirkoy Research and Training Hospital, Istanbul, Turkey
A Ciltas, Department of Gastroenterology, Bakirkoy Research and Training Hospital, Istanbul, Turkey
Correspondence to: A Soylu, MD, Bakirkoy Egitim ve Arastirma Hastanesi, Ic Hastaliklari Klinigi, Istanbul, Turkey. aliyesoylu@superonline.com
Telephone: +90-212-5426969 Fax: +90-212-5713278
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Abstract

Gossypiboma is the technical term for a retained surgical sponge. Because of legal-ethical concerns, there have not been many publications on this topic. Delays in diagnosis and treatment might increase mortality and morbidity. Radiological imaging is used in diagnosis. We present a case of gossypiboma that had fistulized to bulbous following hydatic cyst surgery. We established the diagnosis with endoscopy and followed its migration endoscopically.

Key words: Gossypiboma; Retained surgical sponge; Endoscopy

Alis H, Soylu A, Dolay K, Kalaycı M, Ciltas A. Surgical intervention may not always be required in gossypiboma with intraluminal migration. *World J Gastroenterol* 2007; 13(48): 6605-6607

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INTRODUCTION

Gossypiboma (textiloma, gauzoma, muslinoma) is a rare iatrogenic mass caused by the retention of gauze fibers during surgery. The retention of surgical sponges in body cavities is a rare clinical condition which is preventable[1,2]. Retained surgical sponge or gossypiboma in the abdominal cavity is an infrequent but serious surgical complication that may lead to medicolegal problems. The condition has not been very frequently reported due to possible medicolegal concerns[1,3-5].

CASE REPORT

A 44-year-old woman was admitted with a complaint of abdominal pain that was present for the previous 15 d and was increasing in intensity, together with nausea. In her history she mentioned being operated on 2 mo previously for hydatic cyst, with cystotomy and drainage (Figure 1). On physical examination, there was epigastric pain and tenderness, and laboratory findings were not remarkable, other than for mild leukocytosis. Gastroduodenoscopy was performed for her gastric complaints. Upon endoscopy, a foreign body (gauze) that protruded from the pylorus to the antrum was identified. The gauze could not be retrieved after being approached by biopsy forceps and a polypectomy snare (Figure 2). In upper abdominal CT, there was a foreign body (3 cm × 3.5 cm) with intramural localization in the first and the second parts of the duodenum. It was surrounded by a thin wall, had a density with-600-700 HU (air density/air bubble), heterogeneous internal structure and was protruding in to the duodenum (Figure 3). As there was no free perforation, the decision was taken for conservative treatment, and proton pump inhibitors and liquid diet were recommended. The patient had a stable clinical course and was endoscopically followed-up at 5-d intervals. During the follow-up, the gauze slowly migrated to the lumen. On the fourth endoscopic examination, there was no gauze in the lumen, and there was an ulcerated area with a fistula opening in the middle at the same position (Figure 4). There was no foreign body observed on control CT (Figure 5). Upon questioning the patient, no information could be obtained about the passage of the gauze. Following 3 mo of medical treatment, all the symptoms were gone; upon control endoscopy, ulcer scar was observed on the bulbous.

DISCUSSION

The incidence of surgical sponge being retained during operation is difficult to estimate, but it has been reported as 1 in 100-3000 for all surgical interventions and 1 in 1000-1500 for intra-abdominal operations[4]. Retained sponges are most frequently observed in patients with obesity, during emergency operations[6] and following laparoscopic interventions[7]. Gossypiboma is most frequently diagnosed in the intra-abdominal cavity; however, it can also be seen in paraspinal muscles[8] and the intrathoracic region[9], legs[10] and shoulders[11].

Gossypiboma results in significant morbidity and

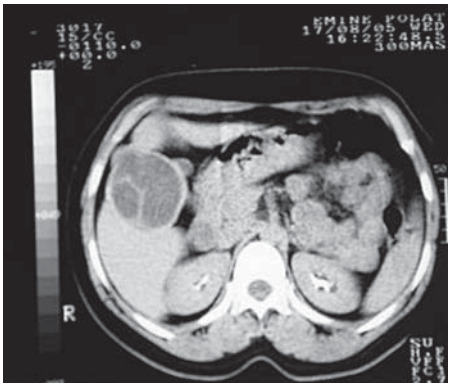

Figure 1 Active hydatic cyst lesion in the liver seventh segment.

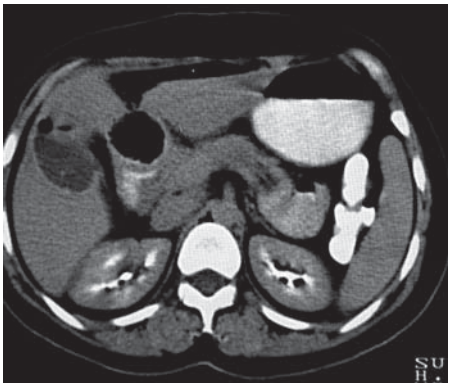

Figure 3 Lesion with a density-600-700 HU (air bubble) and heterogeneous internal structure, hypodense area in the liver pertaining to a postoperative cavity

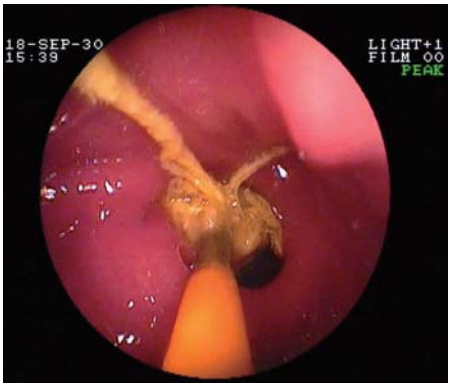

Figure 2 Gauze that could not be removed, despite being approached by biopsy forceps.

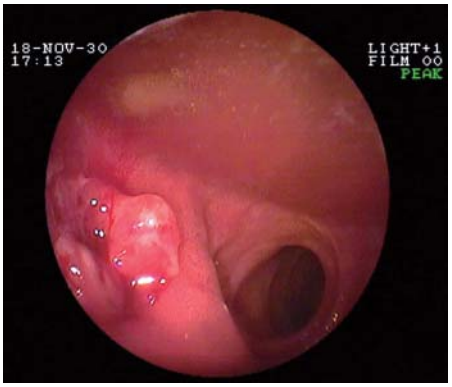

Figure 4 Fistula opening on the anterior part of the bulbous, and surrounding granulation tissue after spontaneous migration of the gauze.

possible mortality[5,12]. It can present itself as an intra-abdominal mass and might lead to erroneous biopsy attempts and unnecessary manipulations[13]. It commonly leads to misdiagnosis and unnecessary surgery[12]. Clinical presentation may be acute or subacute, and may follow months or even years after surgery[14]. Presentation of gossypiboma may vary and can be caused by pseudotumoral, occlusive or septic syndromes[5]. Of 14 patients with a diagnosis of gossypiboma, 13 were admitted with non-specific abdominal pain and intestinal obstruction. Four patients required emergency surgery due to intestinal obstruction or intra-abdominal sepsis[15]. Six patients with abdominal gossypiboma had symptoms of a mass, nausea, vomiting, abdominal distention and pain. Three patients were diagnosed with intestinal obstruction and two with pseudotumoral syndrome[16]. If the patients do not recover in the postoperative period and are readmitted with extraordinary problems, gossypiboma should definitely be considered as the differential diagnosis in such cases[12]. During the period that gossypiboma remains in the body, extrusion of the gauze can occur externally through a fistulous tract or internally into the rectum, vagina, bladder or intestinal lumen[17]. By either fistulizing to a luminal organ or through direct migration, it can cause intestinal obstruction[18-21]. There are reports of spontaneous migration of the sponge from the colon or rectum in the literature[3,6].

There has also been a case report demonstrating the migration of endoscopically confirmed intra-abdominal gossypiboma through fistulization to a luminal organ. In addition to assisting with a definitive diagnosis, endoscopy can also be helpful in planning treatment[18]. The gauze that

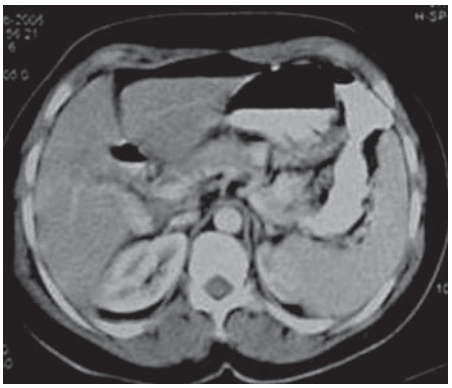

Figure 5 Control CT following the intraluminal migration of the intramurally localized gauze in the first and the second parts of the duodenum.

was endoscopically diagnosed in our case was found to have been corrected in the endoscopic follow-up, without necessitating surgical intervention.

They are mostly diagnosed by radiological methods in clinical practice. In radiological examinations, they are mostly seen as radio-opaque material, yet radiolucent material like sponges can cause diagnostic problems[3]. Plain radiographs suggest the diagnosis if a textile foreign body is calcified, that is, is equipped with a radio-opaque marker, or when a characteristic "whirl-like" pattern is present[22]. In the presence of radio-opaque markers, retained surgical sponges can be easily diagnosed by direct abdominal radiography; yet, if they penetrate and migrate inside the small bowel or bladder, it is difficult to localize them[20]. US images can be classified into two groups, a cystic type and a solid type[19]. US that shows a hyper-reflective mass with a hypoechoic rim, along with a strong posterior shadow, and CT that reveals a whirl-like spongiform pattern

in a hypodense mass, with a thick peripheral rim, are considered the mainstay of investigation[5]. CT and US are necessary procedures in chronic cases, since the lesion may mimic a mass. CT usually reveals a hypodense mass with a thick peripheral rim[22]. Upper abdominal CT examination of our patient revealed a hypodense lesion surrounded by a regular capsule.

Surgery is the preferred method of treatment for gossypiboma. Of eight patients who were diagnosed 12 mo after initial surgery, seven were treated surgically and one was cured with spontaneous migration from the rectum[6]. One patient who developed perforation and an abscess because of migration to the ileum required surgery[7]. There have been rare case presentations like our own, in which total migration to a luminal organ results in recovery without requiring a second operation. However, we think that in the absence of free perforations, as in our case, and in the presence of migration to luminal organs, conservative treatment with clinical and radiological follow-up should be considered.

In order to prevent these types of complications, we need to abide by the rule of total control of all surgical material before and after surgery, which is the main principle in all procedures. Strict measures must be taken to prevent this complication.

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CASE REPORT

Imatinib-induced fatal acute liver failure

Ezequiel Ridruejo, Roberto Cacchione, Alejandra G Villamil, Sebastián Marciano, Adrián C Gadano, Oscar G Mandó

Ezequiel Ridruejo, Roberto Cacchione, Sebastián Marciano, Oscar G Mandó, Hepatology Section, Hematology Section, Department of Medicine, Centro de Educación Médica e Investigaciones Clínicas "Norberto Quirno", CEMIC, Buenos Aires, Argentina
Alejandra G Villamil, Adrián C Gadano, Liver Unit, Hospital Italiano de Buenos Aires, Buenos Aires, Argentina
Correspondence to: Ezequiel Ridruejo, Hepatology Section, Department of Medicine, Centro de Educación Médica e Investigaciones Clínicas "Norberto Quirno", CEMIC, Avda. Las Heras 2939, Buenos Aires, Argentina. eridruejo@cemic.edu.ar
Telephone: +54-11-48091980 Fax: +54-11-48091992
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Abstract

Imatinib mesylate is a drug that has been approved for treatment of chronic myeloid leukemia (CML) in blast crisis, accelerated or chronic phase, and also for advanced gastrointestinal stromal tumors. Severe hepatic toxicity and three deaths from hepatic failure have been reported. We report the case of a 51-year-old woman who was admitted to our institution with severe acute hepatitis. She was diagnosed with CML and began treatment with imatinib mesylate at a dose of 400 mg/d. Five months after beginning treatment, she developed severe hepatitis associated with coagulopathy, and was admitted to our institution. She had been consuming acetaminophen 500-1000 mg/d after the onset of symptoms. She had a progressive increase in bilirubin level and a marked decrease of clotting factor Ⅴ. Five days after admission, grade Ⅱ encephalopathy developed and she was referred for liver transplantation. Her clinical condition progressively deteriorated, and 48 h after being referred for transplantation she suffered a cardiac arrest and died. This report adds concern about the possibility of imatinib-mesylate-induced hepatotoxicity and liver failure, particularly in the case of concomitant use with acetaminophen. Liver function tests should be carefully monitored during treatment and, with the appearance of any elevation of liver function tests, treatment should be discontinued.

Key words: Imatinib mesylate; Hepatotoxicity; Acute liver failure; Liver transplantation

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INTRODUCTION

Imatinib mesylate (Gleevec; Novartis, East Hanover, NJ, USA) is a drug that targets *bcr-abl* tyrosine kinase, an enzyme that is regarded as the cause of Philadelphia-chromosome-positive chronic myelogenous leukemia (CML). It induces a much higher rate of complete cytogenetic remission (CCR), with improved tolerability and better progression-free survival compared to other therapies. It has been approved for treatment of CML in blast crisis, accelerated or chronic phase[1,2], and also for advanced gastrointestinal stromal tumors[3].

Severe hepatic toxicity has been reported in clinical trials. This includes grade 3 (5-20 times ULN) or 4 (> 20 times ULN) transaminase elevation in 1%-5.1% of patients, and grade 3 (3-10 times ULN) or 4 (> 10 times ULN) bilirubin elevation in 0.4%-3.5% of patients. Hepatotoxicity is usually resolved with imatinib dose reduction or interruption. Yet, permanent imatinib discontinuation for hepatic toxicity has been required in 0.5% of patients[4-6]. Three deaths from hepatic failure have been reported: two during treatment for CML (one in a phase 2 clinical trial[4,5] and the other during regular treatment[7]) and one during treatment for polycythemia vera[8]. We report another case of fatal acute hepatic failure in a patient receiving imatinib for CML.

CASE REPORT

A 51-year-old woman was admitted to our institution with severe acute hepatitis. She was diagnosed with CML 7 mo before admission. She was initially treated with hydroxyurea for 1 mo and then began treatment with imatinib mesylate at a dose of 400 mg/d. Five months after starting treatment, she developed asthenia. Laboratory tests showed elevated aminotransferases with normal bilirubin (Table 1). Treatment with imatinib was discontinued. Liver function tests worsened and she developed jaundice (Table 1). Fourteen days after imatinib was discontinued she was admitted to our institution with severe hepatitis associated with coagulopathy (prothrombin time 30% and clotting factor V level 19%) (Table 1). On admission she was jaundiced, had no signs of chronic liver disease and no evidence of encephalopathy. Abdominal ultrasound showed a reduced-size, homogeneous liver. The spleen was normal, and there were no signs of biliary

Table 1 Laboratory values

Date	AST	ALT	ALP	TB	DB	WBC	Neu	PT (%)
29/12/5	70	55	197	0.5	0.14	247	148.2	
21/2/6	9	13	330	0.3	0.08	5.78	4.769	
10/5/6	38	49	204	0.47	0.1			
31/5/6	1060	1493	288	0.68	0.2			
14/6/6	2224	3185	648	8.43	5.45			
16/6/6	2595	3028	668	12.19	7.65			30
21/6/6	1887	1941	831	25.4	19.81	9.86	7.701	13
22/6/6	1626	1757	756	26	20.28	9.47	7.59	11
23/6/6	1495	1752	793	27	20.79	10	7.36	9
24/6/6	1467	1767	800	26.8	21.44	14.4	11.16	9
25/6/6	1359	1553	789	32	23.36	17	12.75	6
26/6/6	1212	1121	774	36	26.64	22.2	17.1	6
27/6/6	1024	900	686	24.7	9.5	86.8	39.9	N-M[1]
28/6/6	1269	561	362	11.5	4.8	42	33.6	N-M[1]

TB: Total bilirubin, n < 12 mg/L; DB: direct bilirubin, n < 3 mg/L; AST: Aspartate aminotransferase, n < 40 IU/L; ALT: Alanine aminotransferase, n < 35 IU/L; ALP: Alkaline phosphatase, n < 240 IU/L; GGT: Gamma-glutamyltransferase, n < 32 IU/L; WBC: White blood cells, n = 4000-10 000 cells/mm^3; Neu: Neutrophils, n = 2000-6500 cells/mm^3; PT: Prothrombin time, n = 70%-100%; [1]N-M: Non-measurable due to extremely prolonged prothrombin time.

obstruction. Portal and suprahepatic veins were patent, with adequate blood flow. Minimal ascitic fluid was observed.

The patient had no known risk factors for viral or alcoholic liver disease. She had not recently used any dietary products or herbal remedies. She had been consuming acetaminophen 500-1000 mg/d after the onset of symptoms. Screening was negative for viral (HAV, HBV, HCV, CMV, EBV, HSV I and Ⅱ) and autoimmune hepatitis (ANA, ASMA, ANCA, LKM1 negative). Urinary copper level was normal, and she had a mild iron overload.

Concerning her CML, status detection of t (9; 22) BCR ABL (p 210) was performed by RT-PCR. It tested positive in peripheral blood and in the bone marrow. During the following days, the patient had a progressive increase of bilirubin level and a marked decrease of clotting factor V (Table 1). Five days after admission, grade Ⅱ encephalopathy developed. Prothrombin time was 6% and clotting factor V level was 10%, with a bilirubin level of 360 mg/L. She was referred for liver transplantation.

On arrival at the transplantation unit, the patient was admitted to the intensive care unit. Encephalopathy progressed to grade Ⅲ. During the following 24 h, she developed multiorgan failure with hypotension, low urinary output, respiratory distress and metabolic acidosis. Vasopressors were required, and high dose steroids (60 mg/d per nasogastric tube) and broad-spectrum antibiotics were started empirically. She was intubated and mechanical ventilation was initiated. Blood, urinary and ascites cultures were negative. Neutrophil count in peritoneal fluid was < 250/mm^3. No evidence of major bleeding was observed. Her clinical condition progressively deteriorated, and 48 h after admission she suffered a cardiac arrest that was unresponsive to resuscitation, and died.

DISCUSSION

Imatinib mesylate is a selective tyrosine kinase inhibitor that is used in CML, Philadelphia-positive acute lymphoblastic leukemia, and also gastrointestinal stromal tumors. Grade 3 or 4 transaminase elevation has been reported in up to 5.1% of patients in phase 2 and 3 clinical trials[4-6]. There have been three reported cases of fatal acute liver failure. In a phase 2 clinical trial, one death was suspected to be related to treatment in a patient taking 600 mg/d. The patient had received a bone marrow transplant and had been concomitantly taking 3000-3500 mg/d acetaminophen 1 mo before starting treatment. The patient died 12 d after beginning treatment[5]. A 46-year-old woman with CML developed abnormal liver function tests and subsequent acute liver failure after 18 mo of treatment with 400 mg/d. She received a liver transplant but later died due to sepsis. The explanted liver had histological features of severe hepatic necrosis[7]. In the third case, a 61-year-old woman with polycythemia rubra vera in spent phase/myelofibrosis received treatment with 400 mg/d 7 wk. She died 6 d after admission, secondary to extensive hepatic necrosis. Post-mortem histology revealed microthrombi within the vasculature of the liver, lungs and spleen. The authors postulated that the mechanism of hepatic necrosis was due to an exacerbation of the underlying prothrombotic tendency of polycythemia vera, which is not present in CML[8].

There are other references to liver toxicity with imatinib, yet all of them resolved after discontinuation of treatment. Data about liver histology varies between reports of focal necrosis with lymphocytic infiltration[9,10]; from marked periportal necrosis with mixed lymphocyte, neutrophil and plasmocyte infiltration[13]; to massive hepatic necrosis[11] or cytolytic acute hepatitis[12]. The time between beginning treatment and development of liver toxicity varies from 11 d to 49 wk[9-12]. In one study, treatment was reinitiated twice after normalization of laboratory tests. In both cases of re-challenge, including one with 2.5% of the current therapeutic dosage, liver toxicity reappeared[13]. See Table 2 for a comparison of previously reported cases of serious (fatal and non-fatal) drug-induced liver damage following treatment with imatinib mesylate (adapted from the report of Cross *et al*[7]).

Data about management of imatinib liver toxicity are scarce. Ferrero *et al* have reported that corticosteroids at low to intermediate dosage can reverse imatinib-induced hepatotoxicity. The use of prednisone (25-37 mg/d) or methylprednisolone (40 mg/d) resulted in the normalization of aminotransferase levels in 2-4 wk in all five patients treated. Imatinib therapy was then resumed at increasing dosage while corticosteroids were gradually tapered, without reappearance of liver toxicity[14].

Novartis, the manufacturer of imatinib, recommends in the package insert of Gleevec that liver function tests (transaminases, bilirubin, alkaline phosphatase and prothrombin time) should be monitored before initiation of treatment and then monthly, or as clinically indicated. If elevation in bilirubin is > 3 × the institutional upper limit of normal (IULN) or if liver transaminase is > 5 × IULN, then Gleevec should be withheld until bilirubin levels have returned to < 1.5× IULN and transaminase has returned to < 2.5 × IULN. Deininger *et al* have recommended obtaining liver function tests before

Table 2 Summary of previously reported cases of serious (fatal and non-fatal) drug-induced liver damage following treatment with imatinib mesylate

Reference	Diagnosis	Time to hepatic dysfunction	Liver histology	Outcome of liver enzymes
Ohyashiki *et al* (2002)	CML	12 d	Focal necrosis of hepatocytes	Resolution after stopping drug
Lin *et al* (2003)	Polycythemia vera	7 wk	Hepatic necrosis	Patient died fatal acute hepatic necrosis
James *et al* (2003)	CML	49 wk	Acute severe cytolytic hepatitis. With necrosis and mild cholestasis	Resolution after stopping drug
	CML	22 wk	Acute cytolytic hepatitis with spotty and some piecemeal necrosis	Resolution after stopping drug
Kikuchi *et al* (2004)	CML	36 wk	Hepatic necrosis	Resolution after stopping drug
Ayoub *et al* (2005)	CML	2 yr	Portal and lobular inflammation. Bridging and multifocal lobular necrosis	Resolution after stopping drug
Cross *et al* (2007)	CML	77 wk	Severe necrosis with multilobular confluent cell dropout and reticulin collapse, multinodular regeneration	Patient died 10 d after liver transplantation
Ridruejo *et al* (2007)	CML	22 wk	Not available	Patient died fatal acute hepatic necrosis

treatment is started, every other week during the first month of therapy, and at least monthly thereafter[15].

Acetaminophen is widely known as a cause of acute liver failure[16] and has been implicated as a significant co-factor in the pathogenesis of acute liver failure in patients with acute hepatitis B and in those taking antitubercular therapy[17]. Even though there is some controversy regarding the safety of acetaminophen in patients treated with imatinib, Deininger *et al* have recommended that patients be advised to use it with caution[15].

Acute liver failure is a rare condition that requires prompt evaluation for liver transplantation[18]. Pretransplant evaluation is required to exclude contraindications, such as malignancy[18,19]. Acute liver failure in patients with potentially treatable or curable cancer, such as hematological malignancy, is extremely unusual. The role of liver transplantation in these cases is unknown. The decision must be individualized to each patient, and discussed between the liver transplant and oncology teams.

The present case report confirms the possibility of imatinib-mesylate-induced liver failure, particularly in the case of concomitant use with acetaminophen. Liver function tests should be carefully monitored during treatment, and with the appearance of any elevation, treatment should be discontinued. Corticosteroids might be an option for treatment in selected cases.

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MEMORIAL

The love of a beloved hapatologist, Dr. Rudi Schmid

You-De Chang

You-De Chang, *World Journal of Gastroenterology*, The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center No.62, Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Correspondence to: You-De Chang, PhD, Science Editor, *World Journal of Gastroenterology*, The WJG Press and Beijing Baishideng BioMed Scientific Co., Ltd., Room 903, Building D, Ocean International Center No.62, Dongsihuan Zhonglu, Chaoyang District, Beijing 100025,
China. y.d.chang@wjgnet.com
Telephone: +86-10-85381901 Fax: +86-10-85381893
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Rudi Schmid, PhD, 1922-2007. Dr. Rudi Schmid, a leading academic physician and scientist in the field of hepatology died in his sleep on Saturday, October 20th, in Kentfield, California. He was 85.

Dr. Schmid was a former dean of the University of California, San Francisco School of Medicine and a liver specialist whose early studies of porphyrins led to several significant fundamental discoveries. He was the first to demonstrate that there were different forms of porphyria. He also developed the first experimental model for hepatic porphyria, a model that for many years was used for most of the work in this field and cited in hundreds of papers. His contributions were recognized when he was awarded the 1990 Friedenwald Medal by the American Gastroenterological Association, its most coveted award for lifetime achievement.

While taking a brief scientific detour from hepatology, he identified the enzymatic defect in McArdle's disease, a hereditary muscle disease, and defined its pattern of inheritance in a family in California's Central Valley.

Dr. Schmid's career as an academic administrator was equally distinguished. Recruited to UCSF in 1966 as a professor of medicine, he was the architect of one of the leading centers for gastroenterology and hepatology research, education and clinical care in the country.

He served as dean of the UCSF School of Medicine from 1983 to 1989. He often cited as his most important achievement as dean helping the faculty understand their crucial role as teachers of medical students. It was his insight that the pace of knowledge generation in basic science was far outstripping the ability of clinicians to understand and apply it in the patient setting. He strongly supported training medical students in the thinking and tools of fundamental science so that as clinicians they could understand the mechanisms and causes of disease. By the end of his deanship scientists such as Nobel laureate J. Michael Bishop were being recognized by the students as much for their teaching as their science.

Dr. Schmid's contributions to academic medicine continued after his deanship. As associate dean for international relations in medicine and pharmacy at UCSF, he particularly focused on developing formal mechanisms for student and faculty exchanges with China, which was starting to be receptive to overtures from the West. Through the Cheng Scholars Program, which he had persuaded his friend, Dr. YT Cheng of Hong Kong, to support, he built a bridge of scientific training and collaboration that is still an active program at UCSF.

His exquisite appreciation of scientific excellence translated to unprecedented support of the basic science graduate programs at UCSF, enabling them to become among the best in the nation. He fostered an environment of interdisciplinary collaboration which is a hallmark of UCSF to this day.

During his tenure as dean he had the unusual opportunity to appoint the chairs of 11 departments, many of whom went on to extremely distinguished careers within UCSF and at the national level. These include Dr. Bruce Alberts, president emeritus of the National Academy of Sciences; Dr. Zach Hall, director emeritus of the National Institute for Neurological Disorders and Stroke; and Dr. Haile T. Debas, chancellor emeritus of UCSF.

As dedicated and serious as he was about his work, Dr. Schmid was also known for his athleticism, passion for travel and sense of fun. A skier and mountain climber for most of his life, as a young man he was on the Swiss National Ski Team and made a number of first ascents in Europe and Peru. His approach to life was deeply influenced by a poem describing the meaning of "youth" given to him as a boy by his father. In it, Samuel Ullman

describes youth as ". . . not a time of life; it is a state of mind . . . it is a matter of the will, a quality of the imagination, a vigor of the emotion . . . the temperamental predominance of courage over timidity of the appetite, for adventure over the love of ease . . ." Those who knew Dr. Schmid say that this described him exactly.

Born in 1922 in Glarus, Switzerland, to physician parents, he studied medicine in Switzerland and completed his medical training and earned his PhD at the University of Minnesota. Dr. Schmid originally vowed to pursue anything but medicine and later said that he supposed he went to medical school because he didn't know anything else. Although he originally came to the Americas after medical school to climb mountains, a series of fortuitous events directed his career first to UCSF, then to Minnesota, the NIH, Harvard, and Chicago and back to UCSF.

Against his father's wishes, he stayed in the US. In an interview several years ago, Dr. Schmid said, "America was unbelievably good to me. It was a country after the Second World War where anybody who had talent and a willingness to work and to fight intellectually could succeed." He was describing himself in that statement.

Among the many honors Dr. Schmid received in his lifetime, most notable are election to the National Academy of Sciences, the Institute of Medicine, the American Association of Arts and Sciences, and Leopoldina, the German Academy of Sciences. In 2005 he was awarded the UCSF Medal, its highest honor.

Served as an honorable Editor-in-Chief of WJG since 2005, Dr. Schmid had given very valuable comments and peer reviews to 24 articles related to hepatology, almost one article every month. As an esteemed hepatologist, Dr. Schmid was invited to take part in the World Chinese Congresson of Digestology sponsored by WJG in October 1998. He gave a very interesting lecture,Gastroenterology in the next century: megatrends in science and practice. He highly evaluated the success of the congresson, and wrote a letter to Lian-Sheng Ma, Editor-in-Chief of WJG, to discuss some confusing concepts and hot topics for Chinese Gastroenterologists and world wide experts. In the past ten years, Dr. Schmid had provided valuable suggestions and made great contributions to the development of WJG.

The staff, particularly Lian-Sheng Ma, Editor-in-Chief, are very sorrowful to Dr. Schmid's pass away on October 20, 2007. A forever memorial is dedicated to the beloved hepatologist.

Dr. Schmid is survived by Sonja, his wife of 58 years, son Peter Schmid and daughter-in-law Diane of San Francisco; daughter Isabelle, son-in-law Michael Franzen and grandson Alexander.

Donations may be sent to the Rudi Schmid Fund for Gastroenterology c/o Annamaria Flamburis University of California Box 0538 513 Parnassus Ave. San Francisco 94143-0538 or Marin Agricultural Land Trust at www.malt.com, Tahoe Rim Trail at www.tahoerimtrail.com.

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Philip Abraham, Dr. Professor
Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400016, India

Frank A Anania, Professor
Emory University School of Medicine, Division of Division Digestive Diseases, 615 Michael Street, Room 255 Whitehead Biomedical Research Building, Atlanta, GA 30322, United States

Meenakshisundaram Ananthanarayanan, Associated Professor
Department of Pediatrics, Annenberg Bldg, Rm.14-24A, Box 1664, The Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY, 10029, United States

Hitoshi Asakura, Director, Emeritus Professor
International Medical Information Center, Shinanomachi Renga Bldg.35, Shinanomachi, Shinjukuku, Tokyo 160-0016, Japan

Marc Basson, MD, PhD, MBA, Chief of Surgery
John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Patrick Bertolino
AW Morrow Gastroenterology and Liver Centre, Centenary Institute of Cancer Medicine and Cell Biology, Locked Bag No 6, Newtown, NSW 2042, Australia

Inge I Depoortere, PhD
Centre for Gastroenterological Research, Gasthuisberg OandN, bus 701, Leuven 3000, Belgium

William Dickey
Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

Stefano Fiorucci, MD
University of Perugia, Gastroenterologia Policlinico Monteluce, Via E. Dal Pozzo, 06122 Perugia, Italy

Ignacio Gil-Bazo, MD, PhD
Cancer Biology and Genetics Program, Memorial-Sloan Kettering Cancer Center, 1275 York Avenue. Box 241, New York 10021, United States

Mark D Gorrell, PhD, Professor
Centenary Institute of Cancer Medicine and Cell Biology, Locked bag No. 6, Newtown, NSW 2042, Australia

Yik-Hong Ho, Professor
Department of Surgery, School of Medicine, James Cook University, Townsville 4811, Australia

Yoshiaki Iwasaki, Dr
Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1, Shikata-cho, Okayama 700-8558, Japan

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

Kaukinen Katri Maria Kaukinen, MD, PhD
Medical School, FIN-33014 University of Tampere, Tampere, Finland

Emmet B Keeffe, Professor
Stanford University Medical Center, 750 Welch Road, Suite 210, Palo Alto, CA 94304, United States

Leonidas G Koniaris, Professor
Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave, Miami, FL 33136, United States

Peter L Lakatos, MD, PhD, Assistant Professor
1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Thomas Langmann, Associate Professor
University of Regensburg, Institute of Human Genetics, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany

Kurt Lenz, Professor
Department of Internal Medicine, Konventhospital Barmherzige Brueder, A-4020 Linz, Austria

Andrew L Mason, Associate Professor
Andrew Mason, Director of Research, Division of Gastroenterology, University of Alberta, Edmonton, Alberta, T6G 2X8, Canada

McGrath Kevin McGrath, MD
Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center, M2, C wing, PUH, 200 Lothrop St, Pittsburgh, PA 15213, United States

James Neuberger, Professor
Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

Damian Casadesus Rodriguez, MD, PhD
Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

Matti Sallberg, Professor
Division of Clinical Virology, F 68, Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden

Tadashi Shimoyama, MD
Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan

Kyuichi Tanikawa, Professor
International Institute for Liver Research, 1-1 Hyakunen Kouen, Kurume 839-0864, Japan

Takuji Torimura, MD
Second Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume City, Fukuoka 830-0011, Japan

Naomi Uemura, MD, Director
Department of Gastroenterology, International Medical Center of Japan, 1-21-1 Toyama, Shinjyuku, Tokyo 162-8655, Japan

Saúl Villa-Trevio, MD, PhD
Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN (Cinvestav), Ave. IPN No. 2508. Col. San Pedro, Zacatenco, C.P. 07360, México, DF, Mexico

Siegfried Wagner, Professor
Medizinische Klinik II, Klinikum Deggendorf, Perlasberger Str. 41, Deggendorf 94469, Germany

Özlem Yilmaz, PhD
Associate Professor of Microbiology, Dokuz Eylul University, School of Medicine, Department of Microbiology and Clinical Microbiology, Inciralti 35340, Izmir, Turkey

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Meetings

Events Calendar 2007-2009

Meeting Falk Research Workshop: Morphogenesis and Cancerogenesis of the Liver
25-26 January 2007
Goettingen
symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Inflammatory Bowel Diseases 2007
1-3 March 2007
Innsbruck
ibd2007@come-innsbruck.at
www.come-innsbruck.at/events/ibd2007/default.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
Barcelona
easl2007@easl.ch
www.easl.ch/liver-meeting

Meeting SAGES 2007 Annual Meeting -part of Surgical Spring Week
18-22 April 2007
Paris Hotel and Casino, Las Vegas, Nevada
www.sages.org/07program/index.php

Meeting Falk Symposium 159: IBD 2007-Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
Washington-DC
tkoral@asge.org

Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
12-15 June 2007
Lisbon
fca@netvisao.pt

Meeting Falk Symposium 160: Pathogenesis and Clinical Practice in Gastroenterology
15-16 June 2007
Portoroz
symposia@falkfoundation.de

Meeting ILTS 13th Annual International Congress
20-23 June 2007
Rio De Janeiro
www.ilts.org

Meeting 9th World Congress on Gastrointestinal Cancer
27-30 June 2007
Barcelona
meetings@imedex.com

Meeting 15th International Congress of the European Association for Endoscopic Surgery
4-7 July 2007
Athens
info@eaes-eur.org
www.congresses.eaes-eur.org

Meeting 39th Meeting of the European Pancreatic Club
4-7 July 2007
Newcastle
www.e-p-c2007.com

Republic of meeting ISNM2007
The 21st International Symposium on Neurogastroenterology and Motility
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www.isnm2007.org/00main/main.htm

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www.escp.eu.com/index.php

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Meeting Falk Symposium 161: Future Perspectives in Gastroenterology
11-12 October 2007
Dresden
symposia@falkfoundation.de

American College of Gastroenterology Annual Scientific Meeting
12-17 October 2007
Philadelphia

Meeting Falk Symposium 162: Liver Cirrhosis-From Pathophysiology to Disease Management
13-14 October 2007
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Meeting APDW 2007-Asian Pacific Digestive Disease Week 2007
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www.apdw2007.org

15th United European Gastroenterology Week, UEGW
27-31 October 2007
Paris

Meeting The Liver Meeting®2007-57th Annual Meeting of the American Association for the Study of Liver Diseases
2-6 November 2007
Boston-MA
www.aasld.org

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8-11 October 2008
Istanbul

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

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Both personal authors and an organization as author

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900]

No volume or issue

9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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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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